Antitumor effect of the combination of exogenous sphingosine-1-phosphate (S1P), S1P receptor 1 ($S1PR_1$) antibody and carboplatin against human breast cancer cells

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Abstract

Breast cancer is the third most common cancer in Canada. Even though the morbidity and mortality rates have reduced in recent years because of early diagnosis and improved treatments, new treatment methods or therapeutic agents are still needed to deal with cases such as low response rates with triple negative breast cancer, rapid drug resistance, and side effects caused by breast cancer treatment.

Sphingolipids have been reported to play an important role in breast cancer tumorigenesis. Sphingosine-1-phosphate (S1P), a pleiotropic lysophospholipid mediator, can regulate S1P receptor (S1PR)-dependent or S1PR-independent cell proliferation, apoptosis, autophagy, migration, survival, angiogenesis, and differentiation. Previous studies from our laboratory have shown that S1P can selectively cause cell death in breast cancer cell lines MDA-MB-231 and MCF-7.

Our previous studies have shown that S1P exhibits synergistic effects with docetaxel, doxorubicin, and cyclophosphamide in human breast cancer MDA-MB-231 and MDA-MB-361 cells. Since carboplatin is a commonly prescribed DNA alkylating agent and approved for advanced breast cancer, I investigated whether S1P or $S1PR_1$ antibody can enhance the cytotoxic effect of carboplatin towards human breast cancer MCF-7, SK-BR-3, and MDA-MB-231 cells.

In this study, $S1PR_1$ antibody was shown to exhibit cytostatic effect against the MCF-7, SK-BR-3 and MDA-MB-231 cell lines. Co-administration of 4,000 ng/mL of the $S1PR_1$ antibody not only potentiated the cytotoxicity of carboplatin towards the MDA-MB-231 cells but also increased the anti-proliferative effect of S1P towards SK-BR-3 cells. Furthermore, we showed that co-administration of S1P did not sensitize the MCF-7, SK-BR-3 and MDA-MB-231 cells towards carboplatin. In the future, co-administration of 4,000 ng/mL $S1PR_1$ antibody with carboplatin, as well other chemotherapy drugs such as docetaxel, paclitaxel and doxorubicin, will be evaluated against more breast cancer cell lines, and the mechanism on how $S1PR_1$ antibody enhances cytotoxicity of carboplatin on breast cancer cells will be studied.

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Dedication

I dedicate this work to my parents, Rumian Xiao and Huoling Chen, who offered unconditional love and support and have always been there for me.

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List of Abbreviations

Aβ	Amyloid-β
ABC	ATP binding cassette
AC	Adenylate cyclase
AD	Alzheimer's disease
Akt	Protein kinase B
Ang2	Angiopoietin 2
BH3	Bcl-2 homology 3
brms1	Breast carcinoma metastasis suppressor 1
C1P	Ceramide-1-phosphate
cAMP	Cyclic adenosine monophosphate
Cer	Ceramide
CERT	Ceramide transfer protein
CGT	Ceramide galactosyltransferase
CK5	Keratin 5
DAG	Diacylglycerol
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eNOS	Endothelial nitric oxide synthase
ER	Estrogen receptor
ERK	Extracellular-signal regulated kinase
ERM	Ezrin-radixin-moesin
FBS	Fetal bovine serum
GCS	Glucosylceramide synthase
GEF	Guanine exchange factor
GlcCer	Glucosylceramide
GSK-3	Glycogen synthase kinase 3
GSTO1	Glutathione S-transferase omega 1
HDAC1/2	Histone acetylation 1 or 2
HER2	Human epidermal growth factor 2

HPA	Human protein atlas
IL-6	Interleukin 6
IP3	Inositol 1,4,5-trisphosphate
JNKs	c-Jun N-terminal kinases
MRPs	Multi-drug-resistance proteins
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium
PDGF	Platelet-derived growth factor
PEG	Polyethylene glycol
P-gp	P-glycoprotein
PHB2	Prohibitin 2
PI3K	Phosphoinositide 3-kinase
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PLC	Phospholipase C
PR	Progesterone Receptor
PTEN	Phosphatase and tensin homolog
RIP1	Receptor interacting protein 1
ROCK	Rho-associated protein kinase
S1P	Sphingosine-1-phosphate
$S1PR_{1-5}$	Sphingosine 1 phosphate receptor 1-5
SK1/2	Sphingosine kinase subtype 1 or 2
SM	Sphingomyelin
SMase	Sphingomyelinase
Sph	Sphingosine
SPL	Sphingosine-1-phosphate lyase
Spns2	Sphingolipid transporters 2
SPT	Serine palmitoyltransferase
STAT3	Signal transducer and activator of transcription-3
TNBC	Triple-negative breast cancer
TNF-α	Tumor necrosis factor-alpha
TRAF2	TNF receptor-associated factor 2
VEGF	Vascular endothelial growth factor

Introduction

Sphingosine-1-phosphate (S1P) is a common product of sphingolipid catabolism and an important bioactive sphingolipid metabolite. It exerts its biological functions both intracellularly and extracellularly to regulate various physiological and pathophysiological processes [1–3]. The intracellular function of S1P is not clearly understood. There is evidence showing that S1P acts as an intracellular messenger to regulate cell growth, invasion and apoptosis [4–6]. The extracellular function of S1P has been well studied, and S1P employs an "inside-out" signaling mode for its biological functions [7–10]. Briefly, S1P is first synthesized inside cells, and then transported out of the cells to interact with a family of five G protein-coupled sphingosine-1-phosphate receptors ($S1PR_{1-5}$) [9,10]. S1P promotes cell proliferation and survival, inhibits cell apoptosis, and enhances angiogenesis via binding $S1PR_1$ and $S1PR_3$ [9–12]. However, upon binding $S1PR_2$, S1P inhibits cell proliferation and survival and induces cell apoptosis [9,10,13,14]. Extensive studies have shown that the S1P-S1PR signaling axis plays an important role in cancer development and progression[9,10]. Furthermore, antagonism of $S1PR_1$ by FTY720, a potent immunomodulator, caused internalization and desensitization of $S1PR_1$ and inhibited tumor-associated angiogenesis [15]. Thus, downregulation of the S1P-$S1PR_1$ signaling pathway would likely be an effective option to impede cancer development and progression.

In addition, platinum-based chemotherapy drugs are a family of alkylating agents widely used to treat different types of cancer. However, breast cancer does not respond well towards platinum-based drugs. Carboplatin, usually in combination with other chemotherapy drugs, is the only platinum-based drug approved to treat advanced-stage breast cancer [16,17]. Since antagonism of $S1PR_1$ has been observed to enhance the efficacy of doxorubicin [15,18], we decided to evaluate whether the $S1PR_1$ antibody could potentiate the cytotoxic effect of carboplatin towards human breast cancer MCF-7, SK-BR-3 and MDA-MB-231 cells.

1 Breast cancer

Breast cancer is the third most common cancer in Canada, accounting for 13% of all cancers and 25% of cancers in women in 2016.[19] Although clinical outcome has been significantly improved for the breast cancer patients, mortality and morbidity rates remain high for late-stage breast cancer.[20] Furthermore, for triple-negative breast cancer (TNBC) patients who even respond to chemotherapy treatment, the median overall survival still remains less than two years.[21]

1.1 Breast cancer development

Breast cancer commonly refers to breast adenocarcinoma, which accounts for 95% of all breast cancer. Normal human breast is a modified, specialized apocrine gland localized on the anterior chest wall overlying pectoralis major and minor muscles.[22] As shown in Figure 1, it includes mammary glands and connective tissue stroma. The connective tissue stroma is a supportive structure that surrounds the mammary glands. It includes fibrous and fatty components.[22] Breast cancer starts typically from cells of the breasts (mostly cells in the milk duct and lobules) losing genomic stability such as elevation of oncogene expression and silencing of tumor suppressor genes. Although extensive efforts have been made to understand how breast carcinogenesis is initiated, there is still no consensus explanation for it. One hypothesis is "a sequential progression of proliferative changes places the breast at progressively increased risk for invasive carcinoma".[11]

Environmental and biological factors can initiate the development of the breast cancer by causing change and mutation of DNA molecules in breast cells. For example, radiation exposure and cigarette smoking can lead to mutations in the breast cell DNA molecules, and people with inherited mutated genes like BRCA1 and BRCA2 have a much higher chance of developing breast cancer.[24] Also, epidemiological studies have established numerous risk factors for breast cancer.[25,26] For instance, early menarche, late menopause, and nulliparity are all associated with increased risk of breast cancer.[25]

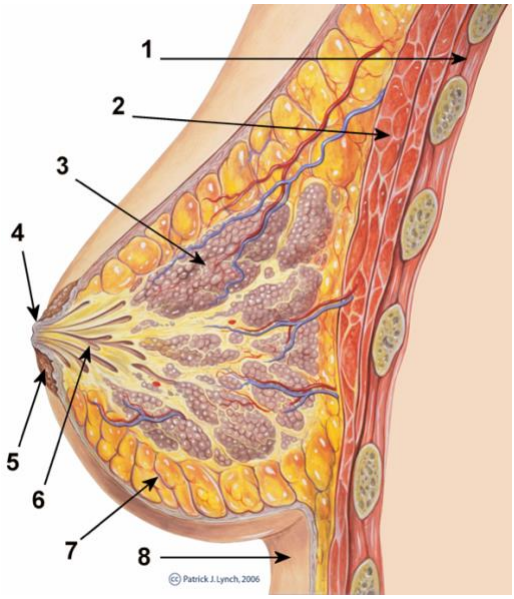


Figure 1: Cross-section scheme of the human mammary gland. 1) Chest wall; 2) Pectoralis muscles; 3) Lobules; 4) Nipples; 5) Areola; 6) Milk duct; 7) Fatty tissue (part of the stroma); 8) Skin.[27] No copyright required.

1.2 Molecular breast cancer subtypes

Breast cancer is a heterogeneous disease. It can be divided into different subtypes depending on the molecular profile, histopathological staining and clinical classification, and associates with different prognostic and therapeutic implications. Based on gene expression profiling, breast cancer is classified into five biologically distinct intrinsic subtypes: luminal A, luminal B, human epidermal growth factor 2 (HER-2), basal-like, and normal-like subtype.[28] Luminal A subtype is breast cancer tumors with estrogen receptor (ER)-positive, progesterone (PR)-positive or PR-negative, HER2-negative, and low proliferating cell nuclear antigen (Ki67) index in immunohistochemistry.[28] It tends to have a higher expression of ER-related genes and a lower expression of proliferative genes compared with luminal B.[29–31] In addition, luminal A subtype shows to have the best prognosis and has significantly lower recurrence rate.[29–31] Luminal B subtype, in immunohistochemistry, is breast cancer tumors with ER-positive, HER2 negative and high Ki67 or ER and HER2 positive.[28] Compared to luminal A subtype, luminal B shows better response to adjuvant chemotherapy, but less sensitive to endocrine therapy.[28] HER2 subtype, most of which are associated with significantly upregulated expression of HER2, is often associated with nodal metastasis.[32] Basal-like subtype which tends to have no

expression of ER, PR, and HER2, has a higher probability of metastasis and death from the progressive disease compared with other subtypes.[32] Triple-negative breast cancer (TNBC) is characterized by lacking expression of ER, PR, and HER2. Therefore, most of TNBC could be classified into basal-like subtype, but not all the basal-like breast cancer subtypes possess triple-negative phenotype.[32] The normal-like subtype is poorly characterized and has been grouped into the classification of intrinsic subtype with fibroadenomas and normal breast samples.[32] They do not express ER, PR, and HER2; therefore, this subtype could also be classified as triple-negative. However, due to low expression of keratin 5 (CK5) and epidermal growth factor receptor (EGFR), they are not generally considered as basal-like breast cancer.[32] There are few studies on normal-like breast cancer, and the clinical significance of this subtype remains undetermined.[28] This classification of breast cancer subtypes helps to divide breast cancer patients into different groups with distinct tumor morphologies and clinical outcomes.[33]

1.3 Treatment

Breast cancer treatment normally includes the following options: surgery, radiation therapy, chemotherapy, hormonal therapy, and targeted therapy. The prognosis and treatment options of breast cancer are generally based on tumor-node-metastasis (TNM) staging,[34] lymphovascular spread, histologic grade, hormone receptor status, HER2 overexpression, comorbidities, and patient menopausal status and age.[35] The typical treatment options for breast cancer are summarized in Table 1. Historically, there was only one option for breast cancer treatment – surgery; however, it could not prevent subsequent recurrences and breast cancer-related deaths.[35] Therefore, breast-conserving treatment and systemic therapy were used to treat breast cancer.[36] Nowadays, most females with breast cancer will be treated with adjuvant systemic therapy which may include endocrine manipulation, chemotherapy, biologic therapy, or their combination.[36] Among different therapies, surgery is the primary method to treat breast cancer. It contains either breast-conserving surgery following preoperative needle localization of the micro-calcifications or total mastectomy.[37] Radiation therapy is a treatment that uses the high dose of radiation to damage DNA and cell membranes of cancer cells.[38] Hormonal therapy uses competitive antagonists to block hormone receptors on the cell surface such as ER or inhibitors of hormones synthesis .[39]

Table 1. Typical treatments for breast cancer by stage[35]

		Adjuvant therapy		
Cancer Stage	**Primary treatment**	**Hormone receptor negative**	**Hormone receptor positive**	**HER2 overexpression**
Stage 0: Lobular carcinoma	no	-	-	-
Stage 0: Ductal carcinoma in situ	Surgery (breast-conserving or mastectomy)	-	-	-
Stage I and II	Induction chemotherapy, breast-conserving surgery, radiation therapy	Chemotherapy	Chemotherapy, endocrine therapy	Chemotherapy and trastuzumab
Stage III: Locally advanced Noninflammatory	Induction chemotherapy, followed by breast-conserving surgery and radiation therapy	Induction chemotherapy	Induction chemotherapy and post-operative endocrine therapy	Induction chemotherapy and postoperative trastuzumab
Stage III: Locally advanced Inflammatory	Induction chemotherapy, followed by mastectomy and radiation therapy			
Stage IV	Radiation therapy or bisphosphonates for bone pain	Chemotherapy	Endocrine therapy with/without chemotherapy	Trastuzumab with/without chemotherapy

Note: Permission to use this table is granted by the American Academy of Family Physicians.

1.3.1 Chemotherapy

Chemotherapy is vital for breast cancer treatment (Table 1). During the last decade,

significant progress has been made in cytotoxic chemotherapy for advanced and early-stage breast cancer treatment.[21] Chemotherapy is a treatment using anti-cancer drugs to destroy dividing cancer cells or stop their division.[40] For patients diagnosed with HER2 positive metastatic breast cancer, trastuzumab alone or with chemotherapy will always be considered as first-line treatment.[41] For patients with hormone receptor-negative plus HER2-negative metastasis breast cancer, single-agent chemotherapy or combination chemotherapy of paclitaxel with bevacizumab will be given as first-line treatment.[41] For patients with hormone receptor-positive and HER2-negative breast cancer, endocrine treatment will first be considered as first-line therapy before chemotherapy.[41,42] The most commonly used first-line chemo-drugs are anthracyclines (such as doxorubicin, epirubicin, pegylated liposomal doxorubicin) and taxanes (such as paclitaxel, docetaxel, and albumin-bound paclitaxel).[41–43] Taxanes are also the most effective second-line treatment for metastatic breast cancer patients who have developed drug resistance for anthracyclines.[44] If patients fail both anthracycline and taxane treatments, other chemo-drugs that possess different mechanisms to destroy cancer cells will be considered as second-line or later-line chemotherapy treatment such as capecitabine, gemcitabine, ixabepilone, and vinorelbine.[44]

1.3.1.1 Carboplatin

Carboplatin is a chemo-drug used to treat different kinds of cancer specifically for testis, ovary, head, neck and small cell lung cancer.[45] It is classified as a DNA alkylating agent that can covalently link to DNA bases and form DNA adducts. Platinum-based chemotherapy is not commonly used in breast cancer treatment, but it could develop into a highly important new treatment modality to TNBC especially against two TNBC subgroups – basal-like 1 and 2, which are characterized by high expression levels of DNA-damage response genes.[16,17] Based on its sensitivity against TNBC, it is possible that platinum-based drugs are used to predict the *in vivo* situation for breast cancer patients.[16] Currently, carboplatin is the only platinum-drug approved for breast cancer treatment by FDA.

Carboplatin was developed to overcome the severe side effects of cisplatin.[45] Cisplatin, one of the platinum-drugs, is an effective antitumor agent. Studies have shown that vinorelbine in combination with cisplatin could be used as salvage treatment for patients with metastatic

breast cancer and developed resistance towards anthracyclines or taxanes.[41,46,47] Although carboplatin has fewer side effects than cisplatin, it is significantly less potent compared with cisplatin and is still associated with the risk of cumulative toxicities that make it limit for cancer treatment.[45] Therefore, finding a method to improve the potency of carboplatin is essential for the development of more treatment options for breast cancer patients.

1.3.2 Targeted therapy

Targeted therapy is a treatment that targets the overproduced growth-promoting proteins of breast cancer cells.[48] It has less severe side effects compared with traditional chemotherapy.[48] Along with the greater understanding of the biology of breast cancer, more molecular targets have been found and more novel therapies developed.[49] Examples include inhibitors of estrogen receptor (ER), human epidermal growth factor receptor 2 (HER2), extracellular-signal regulated kinase (ERK), protein kinase B (Akt) and phosphoinositide 3-kinase (PI3K).[49] Among these targets, ER and HER2 are well-established therapeutic targets for breast cancer. Agents that target ER, such as tamoxifen and aromatase inhibitors, and HER2, such as trastuzumab and pertuzumab, are among the most successful cancer therapeutics.[50] In Figure 2, the ER and PI3K signal pathways are shown to regulate cell proliferation and survival. Currently, these targeted methods are commonly used in combination with chemotherapy.

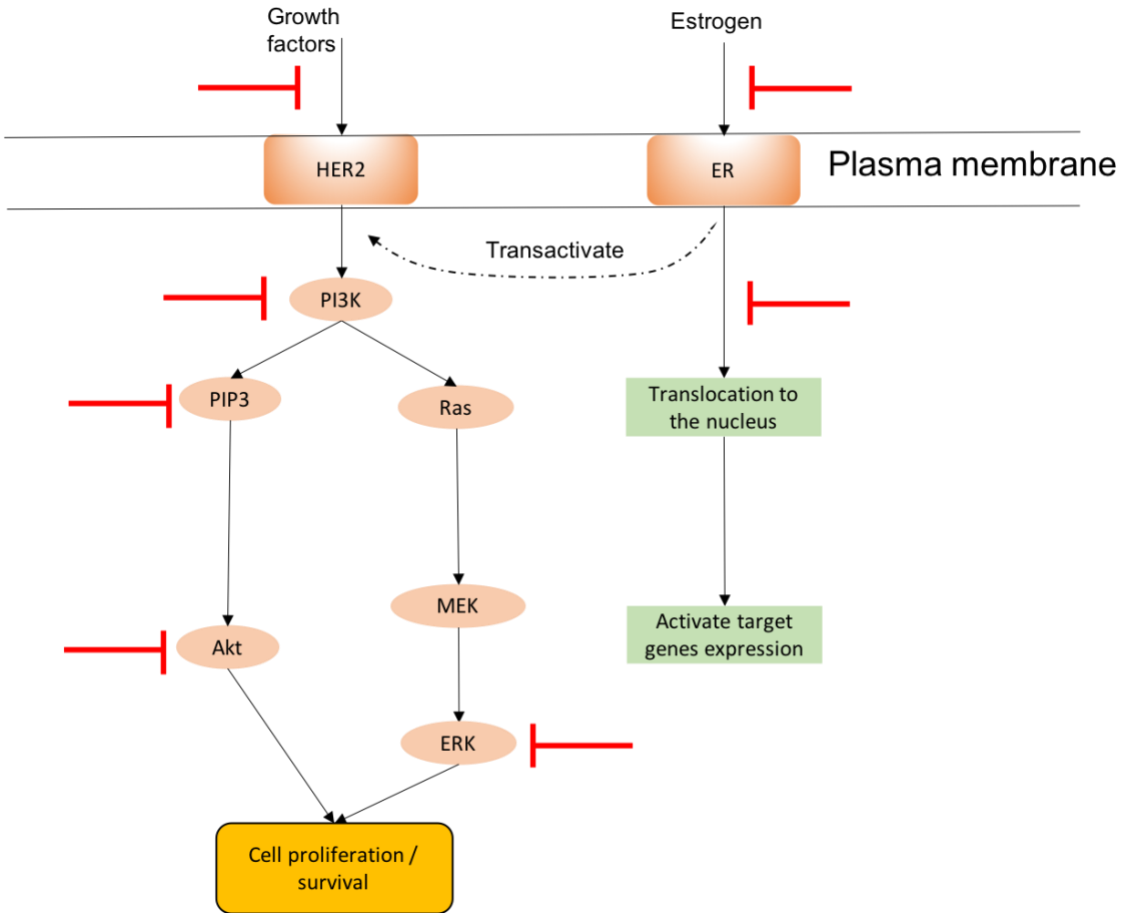


Figure 2. ER pathway and PI3K signaling pathway. Agents used in targeted therapy can inhibit the proteins as the signs in red show.

1.3.3 Challenges for breast cancer treatment

The overall 5-year relative survival rate for female breast cancer patients has improved because of the advances in treatment and earlier detection as the result of increased awareness and widespread use of mammography.[51] However, new treatment methods or treatment agents are still needed because of the low response rates by triple negative breast cancer, drug resistance, and side effects caused by breast cancer treatment.

For breast cancer patients, based on biological, clinical, patient-specific factors, different chemotherapy agents are available for them. However, the response rates of these drugs are not ideal, especially for metastatic breast cancer. For example, anthracyclines and taxanes, the

chemotherapeutic agents possessing a dominant position in breast cancer treatment, the response rate as the first-line therapy for metastatic breast cancer is 38% and 33%, respectively.[41,52]

Drug resistance is another issue that will arise during breast cancer treatment. At the start, agents used in systemic therapy are active in 90% of primary breast cancer and 50% of metastases.[53] After a variable period, though, cancer progresses, and drug resistance is expected to occur.[53] Drug resistance is the cause for more than 90% of treatment failure against metastatic diseases.[54] One mechanism for drug resistance is related to ATP binding cassette (ABC) transporters including P-glycoprotein (P-gp) and multi-drug-resistance proteins (MRPs) which are responsible for transferring drug molecules such as anthracyclines and taxanes out of the cells.[54] Therefore, increased expression of ABC transporters might be found in cells that have developed chemo-resistance. Although several inhibitors of ABC transporters are available, their therapeutic results are not satisfactory.[54] Therefore, discovering and developing new agents that can overcome the mechanisms of multi-drug resistance is essential for improved breast cancer treatment.[54]

Short-term and long-term health effects caused by breast cancer treatment remain a concern.[51] For example, studies have shown that 25% - 60% of women develop chronic pain after breast cancer surgery.[55,56] Chemotherapy treatment can cause impaired fertility and menopause-related concerns such as osteoporosis.[57] Most often, chemotherapy with taxanes leads to neuropathy that can persist long after treatment ends.[58] Also, breast cancer survivors may suffer from cognitive impairments and chronic disease because of the treatments.[51] Therefore, agents with fewer side effects are needed so that better quality of life can be achieved after breast cancer treatment.

2 Sphingolipids

Sphingolipids, a type of lipid found in cell membranes, play an essential role in a wide array of cellular activities by participating in various membrane functions and signaling events.[59–61] They are found in all eukaryotic cells, especially plentiful in the plasma membrane and related cell membranes, such as Golgi membranes and lysosomes.[62] In general, the

chemical structure of sphingolipids is based on an 18-carbon amine alcohol backbone (sphingoid base), linked to a fatty acid molecule *via* an amide bond.[63] Sphingolipids comprise a large family of members, including sphingosine-1-phosphate (S1P), which regulates cellular signal transduction, contributing to the determination of cell fate.[64]

Sphingolipid metabolism wields a double-edged sword to cell fate.[61,64,65] In response to stressful conditions or cytotoxic therapy, ceramide can trigger activation of signals that affect intrinsic and extrinsic apoptotic pathways and cell cycle control, while simultaneously inhibiting signaling events that promote cell growth and survival mediated by the Akt pathway.[64] S1P can promote or inhibit cell growth, survival, angiogenesis, and apoptosis depending on the cell context (S1PRs expression status and the availability of downstream signaling pathways).[61,64,65]

2.1 Sphingolipid metabolism

Two pathways exist to synthesize sphingolipids inside cells -- *de novo* biosynthesis and recycling of sphingomyelin (Figure 3). *De novo* synthesis begins with condensation of serine and palmitoyl-CoA to form 3-ketosphinganine, which is catalyzed by serine palmitoyltransferase (SPT) in the endoplasmic reticulum.[66] Then, 3-ketosphinganine is reduced to form sphinganine, followed by incorporation of a fatty acyl-CoA to form dihydroceramide in a reaction catalyzed by ceramide synthase activity.[67] L-Alanine or L-glycine, two other kinds of amino acid-based sphingolipids, are reported to form 1-deoxy- or 1-deoxy-methyl-derivatives, respectively.[68,69] Dihydroceramide can be converted to ceramide by a desaturase. Once synthesized, ceramide is transported to the Golgi apparatus to synthesize other sphingolipids.[66] Since ceramide has low solubility in an aqueous environment, it needs the help of ceramide transfer protein (CERT) to transport from ER to Golgi apparatus.[66] In Golgi, ceramide is transferred to sphingomyelins (SM) by sphingomyelin synthase and forms glucosylceramide by ceramide galactosyltransferase (CGT) or ceramide-1-phosphate (C1P) by ceramide kinase (CERK).[66] SM, however, can be hydrolyzed back to ceramide by sphingomyelinase (SMase).[66] This is the second pathway to synthesize ceramide. Subsequently, ceramide could be deacylated into sphingosine (Sph) though ceramidases. Sphingosine-1-phosphate (S1P), the last sphingolipid in this cycle, is generated *via* direct phosphorylation of free sphingosine catalyzed by

sphingosine kinases (SK).[66] S1P can be degraded to ethanolamine phosphate and hexadecenal by sphingosine-1-phosphate lyase (SPL) or convert back to sphingosine by sphingosine-1-phosphate phosphatase.[66]

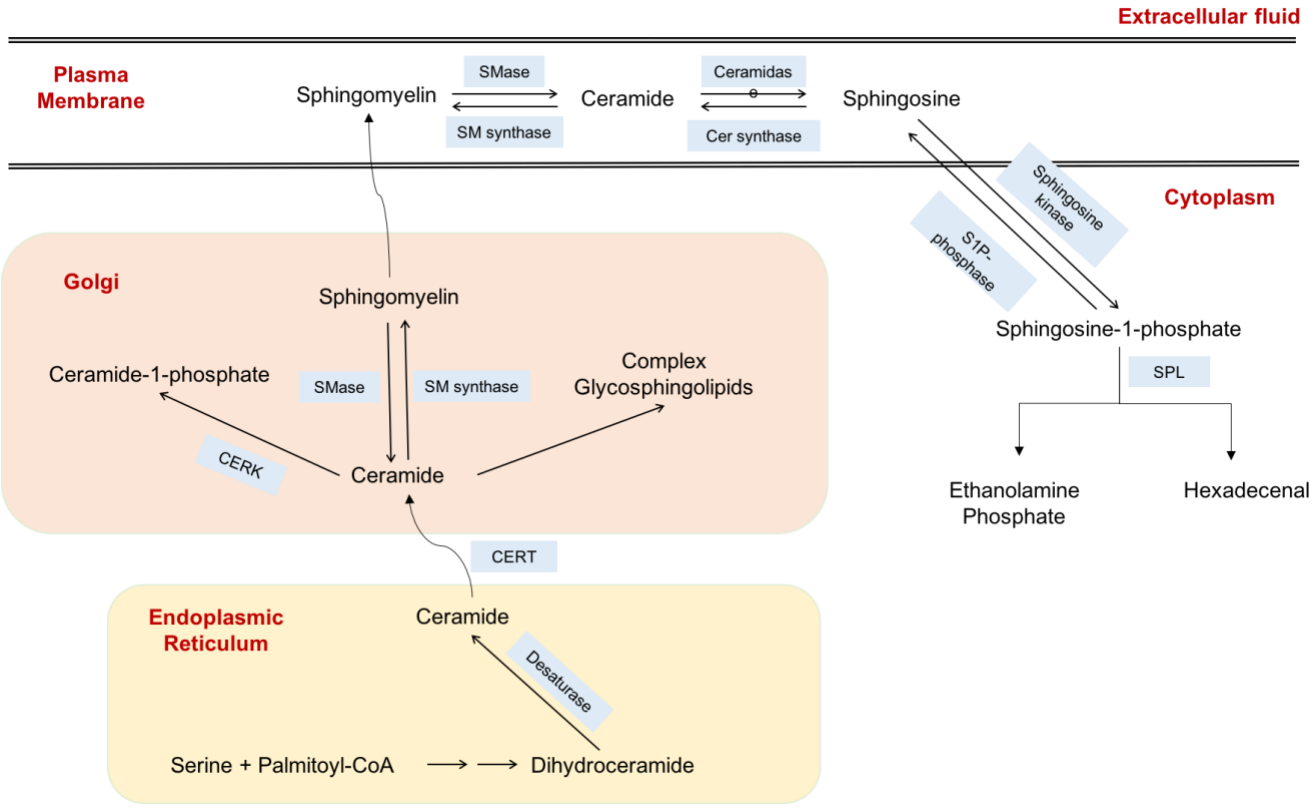


Figure 3. Sphingolipid metabolism. Ceramide (Cer) can be synthesized from *de novo* biosynthesis, which begins in the endoplasmic reticulum with serine and palmitoyl-CoA. Cer is transported to Golgi apparatus by ceramide transfer protein (CERT). In Golgi, Cer is further metabolized to sphingomyelin by sphingomyelin synthase or to ceramide-1-phosphate by ceramide kinase (CERK) or to complex glycosphingolipids. Sphingomyelin (SM) can be converted back to Cer by sphingomyelinase (SMase), which is the second way to synthesize Cer. In the membranes, ceramide can be catalyzed by ceramidases to form sphingosine, which could be further phosphorylated to form sphingosine-1-phosphate (S1P) by sphingosine kinase (SK). S1P could be converted back to sphingosine by S1P phosphatase or degraded by S1P lyase (SPL).

2.2 Sphingosine-1-phosphate

Sphingosine 1-phosphate (S1P), a vital sphingolipid metabolite, plays a crucial role in many physiological and pathophysiological processes, such as cell growth, angiogenesis, and migration.[70] The chemical structure of S1P is shown in Figure 4. S1P is a common product of sphingolipid catabolism that can act directly on intracellular targets or be transported out of the cell and bind to its G protein-coupled receptors, sphingosine-1-phosphate receptors 1-5 ($S1PR_{1-5}$), on the cell surface.[63] Depending on the cell type, location of the receptor, and the specific receptor subtype, S1P will cause cell responses that influence cell survival, proliferation, angiogenesis, chemo-resistance, and migration.

Intracellular and extracellular S1P levels are always under tight control by several enzymes. Specifically, hydrolysis of the sphingolipid complex is controlled by sphingomyelinases and glycosidases.[71] Subsequently, ceramidases can hydrolyze ceramide to produce sphingosine, a direct precursor of S1P by the action of sphingosine kinases.[71] S1P is also regulated by enzymes responsible for its degradation (mainly S1P lyase).[71]

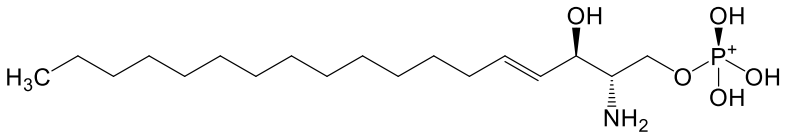


Figure 4. Chemical structure of sphingosine-1-phosphate

2.2.1 S1P inside-out signaling

S1P inside cells cannot freely pass through the plasma membrane because its polar head group impedes passive diffusion. Consequently, to be transported outside of the cells, S1P needs the help of transporters. ATP-binding cassette (ABC) transporters and sphingolipid transporters 2 (Spns2) were found to assume this responsibility.[7,72] Therefore, one way for S1P to exert its function after generation by SK1 is release from the cells and subsequent binding to its receptors on the cell surface through an autocrine, paracrine and/or endocrine manner. [7] This process is called S1P inside-out signaling.[7]

2.2.2 S1P axis

The sphingosine-1-phosphate (S1P) axis refers to the signaling molecule S1P, its

receptors and its intracellular targets, as well as the proteins that synthesize, transport and degrade S1P.[73] In the following section, the functions of different components in the S1P axis will be discussed.

2.2.2.1 Sphingosine kinases

There are two isoforms of sphingosine kinase, sphingosine kinase 1 (SK1) and sphingosine kinase 2 (SK2), which are members of diacylglycerol (DAG) kinase family.[74] Even though they can produce the same product, their catalytic properties, subcellular locations, tissue distribution and expression pattern are different, which, in turn, determines their respective distinct or specific effects inside cells.[63]

SK1, which contains 384 amino acids, is encoded by gene *SPHK1* on chromosome 17q25 with the highest mRNA expression level in lung, spleen, kidney and blood.[75] It is mainly distributed within the cytoplasm.[75] Through translocation to the plasma membrane where sphingosine resides, SK1 can enhance S1P production and extracellular release of S1P.[76,77] SK1 can be activated by numerous growth factors such as platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and tumor necrosis factor-α (TNF-α), and by histamine, vitamin D, and steroid hormones.[75] Previous studies suggest that SK1 and formation of S1P are related to the cell growth and survival.[78] For example, VEGF can stimulate SK1 to produce S1P, which mediates VEGF-induced activation of Ras GTPase, followed by ERK activation and cell growth.[78] Overexpression of SK1 in cells enhances cell survival and allows resistance to agents that can induce apoptosis.[79] In addition, SK1 regulates cancer cell migration through receptor crosstalk.[79] For example, in EGF-induced cell migration of MCF-7 cells, activating SK1 by platelet-derived growth factor (PDGF) signaling stimulated $S1PR_1$ receptor by secreted S1P, inducing Rac GTPase activation.[80,81]

SK2, which contains 618 amino acids, is encoded by gene *SPHK2* on chromosome 19q13.33 with the highest mRNA expression level in liver, kidney, brain, and heart.[75] Unlike SK1 that only uses sphingosine as the substrate, SK2, which is predominantly located in the nucleus or perinuclear region of the cells, utilizes a variety of sphingoid bases as substrates, such as dihydrosphingosine.[82] Furthermore, SK2 possesses not only an overlapping role of SK1 in

promoting tumor development but also an opposing role in inducing apoptosis.[78] SK2 was found to suppress growth and markedly enhance apoptosis that was independent of S1PRs.[78] SK2 contains a functional Bcl-2 homology 3 (BH3) domain, which may allow it to interact with Bcl-2 family members to trigger apoptosis.[78] This may be the mechanism for SK2 to induce cell apoptosis.[78] All in all, overexpression of SK2 could lead to inhibition of cell growth and induction of apoptosis.[4,83–85]

2.2.2.2 Sphingosine-1-phosphate lyase

Sphingosine-1-phosphate lyase (SPL) is a single transmembrane protein that is exclusively localized to the endoplasmic reticulum. The activity of SPL depends on its cofactor pyridoxal-5'-phosphate (PLP), which is ubiquitously found in the ER except in platelets.[75] SPL is responsible for irreversible degradation of phosphorylated sphingoid bases such as S1P, dihydrosphingosine-1-phosphate and phytosphingosine-1-phosphate to hexadecenal and phosphoethanolamine.[66] Therefore, it can control the levels of bioactive sphingolipid metabolites. SPL may be vital for tissue development and associated with some diseases such as Sjögren–Larsson syndrome[86].

2.2.2.3 S1P receptors

The extracellular function of S1P is related to its receptors ($S1PR_{1-5}$) on the cell surface[87–89]. Through binding to different receptors, S1P can cause various downstream reactions. S1PRs belong to the G-protein coupled receptors superfamily. In the human body, the distribution sites of S1P receptors are summarized in Table 2. S1P receptors can couple to different G-proteins. For example, $S1PR_1$ couples only with $G_{\alpha i}$, whereas $S1PR_2$ and $S1PR_3$ couple with $G_{\alpha i}$, $G_{12/13}$ and G_q.[90] Depending on type and abundance of S1PRs, as well as their respective downstream signaling, S1P influences cell survival, cell proliferation, cell apoptosis, cell migration, angiogenesis, chemo-resistance, and other functions.[87–89]

Table 2: Distribution of different Sphingosine-1-Phosphate receptors[91]

S1PR	Distribution	
	Tissue	**Cellular**
$S1PR_1$	Most tissues, highest in CD19+ B cells and cerebellum	Plasma membrane, caveolae, cytoplasmic vesicles, nucleus, perinuclear region
$S1PR_2$	Most tissues	Plasma membrane, cytoplasm
$S1PR_3$	Highest in heart, lung, spleen, kidney, intestine, diaphragm	Plasma membrane
$S1PR_4$	Highly expressed in lymphoid tissues and blood cells, especially CD19+ B cells and lung	N/A
$S1PR_5$	Mostly in brain, skin and natural killer cells	N/A

Note: Permission to use this table is granted by the John Wiley and Sons.

2.2.2.3.1 $S1PR_1$

$S1PR_1$ is ubiquitously expressed in cells. As a pro-tumorigenic receptor, it promotes cell proliferation, survival, migration, angiogenesis, and metastasis (Figure 5).[92–94] In addition, $S1PR_1$ plays a vital role in tumor progression. [95] For example, the previous study has shown that $S1PR_1$ is vital in mediating S1P-stimulated glioma cell proliferation.[95] In $S1PR_1$ knockdown mice, tumor growth is suppressed because of the lack of new blood vessel formation within the tumor.[96]

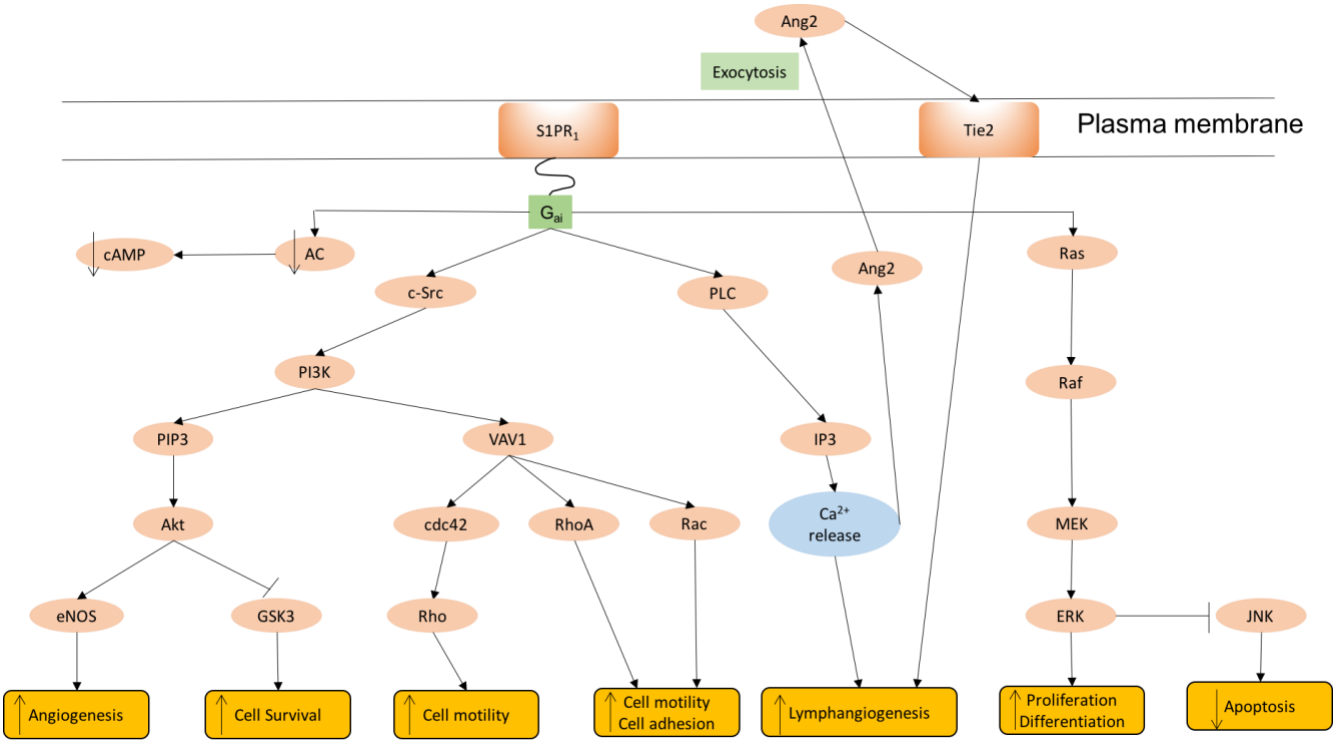


Figure 5. Major signaling pathways and functions of extracellular S1P by binding to $S1PR_1$ on the cell surface. The activation of $S1PR_1$ receptor can reduce AC level, stimulate PI3K, PLC and/or Ras GTPase protein, leading to activation of cell angiogenesis, cell survival, cell proliferation, cell differentiation, cell motility, cell adhesion, lymphangiogenesis, and reduction of cell apoptosis.

With activation by S1P, $S1PR_1$ can initiate different cell signaling pathways through coupling with $G_{\alpha i}$ protein.[97] It is capable of inhibiting adenylate cyclase (AC) activity, and subsequently, significantly reducing cyclic adenosine monophosphate (cAMP) level within the cells.[95] $S1PR_1$ signaling can also activate the PI3K/Akt pathway, eventually leading to angiogenesis and cell survival.[98–100] The maintenance of the cell membrane integrity is through the phosphorylation of endothelial nitric oxide synthase (eNOS) or inhibition of glycogen synthase kinase 3 (GSK-3) to promote cell survival in endothelial cells.[101,102] The activation of PI3K can also enhance cell adhesion and motility by stimulating a member of guanine nucleotide exchange factors (GEF), VAV1, and its downstream factors CDC42, RhoA and Rac.[103] In addition, $S1PR_1$ can work on the Ras-Raf-MEK-ERK pathway to increase cell proliferation and differentiation or to suppress cell apoptosis by inhibiting c-Jun N-terminal

kinases (JNKs).[104] Moreover, phospholipase C (PLC) can be stimulated by $S1PR_1$, inducing lymphangiogenesis because of the mobilization of calcium.[105] Angiopoietin 2 (Ang2) can be secreted from the cells triggered by exogenous S1P *via* the $S1PR_1/G_{\alpha i}$/PLC/calcium signaling pathway, then bind to Tie2 in an autocrine fashion to induce lymphangiogenesis alternatively.[106] Recently, a positive feedback loop was found between signal transducer and activator of transcription-3 (STAT3) and $S1PR_1$.[92,94] STAT3 was found to be a transcription factor for $S1PR_1$.[92] Activated $S1PR_1$ upregulates the expression of interleukin 6 (IL-6), a pro-inflammatory cytokine crucial for STAT3 activation, inflammatory, and cell-mediated transformation, and tumor progression.[98] Therefore, S1P-mediation activation of $S1PR_1$ results in the activation of different signal transduction pathways to enhance cell survival, proliferation, cell motility, angiogenesis, and lymphangiogenesis and inhibit cell apoptosis.

2.2.2.3.2 $S1PR_2$

$S1PR_2$ can be an anticancer receptor or promote cancer growth receptor based on the G proteins it couples to and the available downstream reactions.[91] $S1PR_2$ is vital for normal body functions. *In vivo* studies of $S1PR_2$ knockout mice showed that seizures were detected and occasionally fatal.[107] Furthermore, $S1PR_2$-deficient mice have decreased renal and mesenteric vascular resistance, indicating its function on vasculature.[108]

As Figure 6 shown, $S1PR_2$ can couple to G_{ai}, G_q, or $G_{12/13}$ proteins and activate different downstream reactions. Similar to $S1PR_1$ activation, $S1PR_2$ can cause AC activity and reduction in cellular cAMP levels *via* G_{ai}.[91] $S1PR_2$ also activates Ras-Raf-MEK-ERK pathway through G_{ai}, leading to elevation of cell proliferation.[95] Also, $S1PR_2$ can activate PLC to induce inositol 1,4,5-trisphosphate (IP_3) and Ca^{2+} mobilization, as well as increase cell lymphangiogenesis by G_{ai} proteins.[95] In bladder cancer, $S1PR_2$ was shown to down-regulate tumor suppressor proteins such as breast carcinomas metastasis suppressor 1 (brms1).[109] Therefore, $S1PR_2$ was thought to be a receptor that can enhance tumorigenesis.

$S1PR_2$ can also mediate the stimulation of PLC and Ca^{2+} mobilization *via* G_q protein that can lead to the vascular contraction in some vascular beds, therefore regulates normal blood flow.[110]

Compared with G_q, $S1PR_2$ prefers to couple to $G_{12/13}$. Coupled with $G_{12/13}$, $S1PR_2$ can activate RhoA GTPase and inhibit Ras GTPase activity, causing reduction in cell migration and membrane ruffling.[91] Activating Rho GTPase can lead to activation of Rho-associated protein kinase (ROCK) and phosphatase and tensin homolog (PTEN), a protein that can inhibit Akt activity by dephosphorylating phosphatidylinositol (3,4,5)-trisphosphate (PIP_3), reducing cell proliferation.[102] Therefore, $S1PR_2$ is an anti-tumorigenic molecule and can hinder cells from distant metastasis. $S1PR_2$ was reported to exist inside cells, and endogenous $S1PR_2$ is solely responsible for mediating suppression of migration and invasion by S1P.[14]

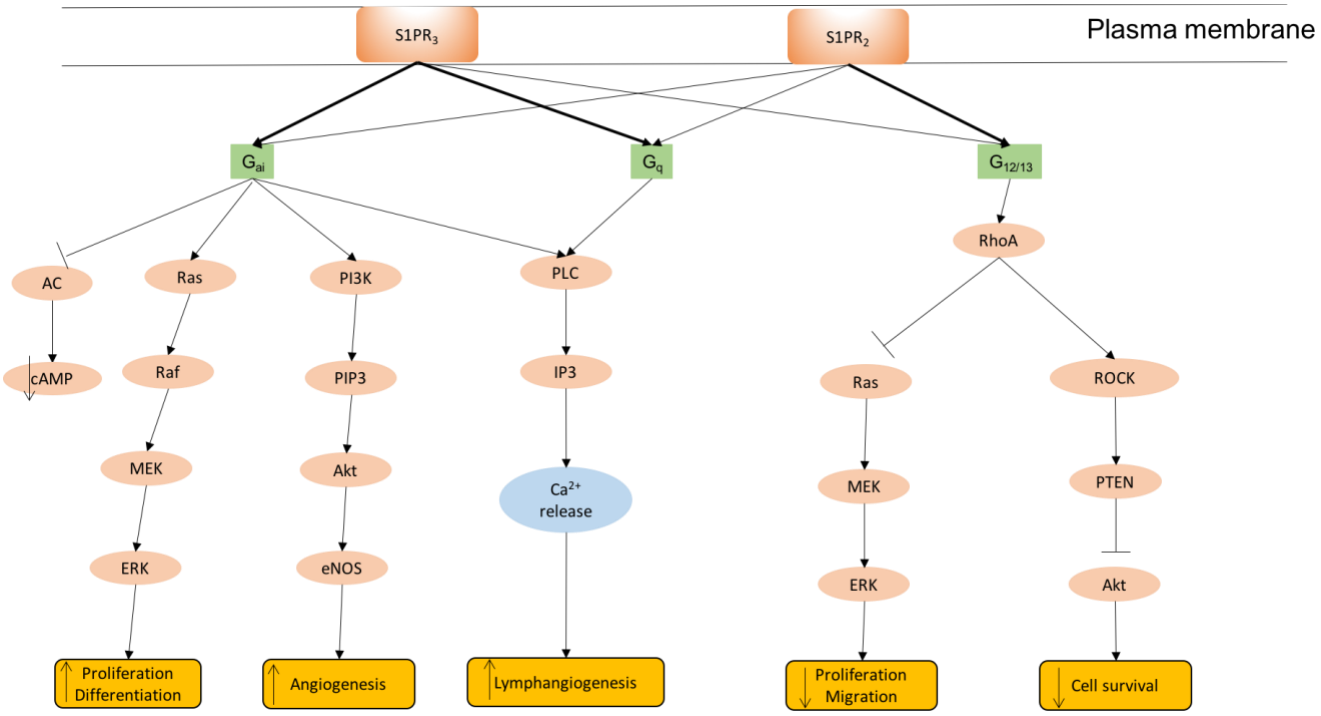


Figure 6. Major signaling pathways and functions of extracellular S1P by binding to $S1PR_2$ or $S1PR_3$ on the cell surface. $S1PR_2$ and $S1PR_3$ can couple to $G_{\alpha i}$, $G_{12/13}$ or G_q proteins. Through the activation of the $G_{\alpha i}$ protein, $S1PR_2$ or $S1PR_3$ can downregulate AC level, stimulate Ras, PI3K and/or PLC protein, leading to the upregulation of cell proliferation, cell angiogenesis, and cell lymphangiogenesis. By activating G_q protein, $S1PR_2$ or $S1PR_3$ activates PLC protein, causing activation of cell lymphangiogenesis. Coupled with $G_{12/13}$ protein, $S1PR_2$ or $S1PR_3$ can inhibit cell survival, cell proliferation and cell migration *via* activating ERK and inhibiting Akt proteins.

2.2.2.3.3 $S1PR_3$

$S1PR_3$ is less studied compared to $S1PR_1$ and $S1PR_2$. $S1PR_3$ knockout in mice does not result in an obvious phenotype.[111] As Figure 6 shown, $S1PR_3$ can couple to $G_{\alpha i}$, $G_{12/13}$ and G_q. When coupling with $G_{\alpha i}$, similar to $S1PR_1$, $S1PR_3$ reduces the cAMP level and activates Ras-Raf-MEK-ERK pathway, resulting in an increase in cell proliferation and an inhibition of apoptosis.[95] With activation by S1P, $S1PR_3$ can trans-activate different receptors.[112–114] For example, in breast cancer, estrogen stimulation could cause $S1PR_3$ to trans-activate EGFR and leads to an increase in cell proliferation.[115]

Among $G_{12/13}$ and G_q proteins, unlike $S1PR_2$, $S1PR_3$ prefers to couple with G_q protein, thus favoring PLC activation.[95] Therefore, it is possible that the effect of S1P might be determined by the balance between $S1PR_2$- and $S1PR_3$- mediated signals.[116] In gastric cancer cells, when the expression level of $S1PR_2$ is higher than that of $S1PR_3$, S1P inhibits cell migration and *vice versa*.[116] Therefore, mostly $S1PR_3$ was observed to elude pro-tumorigenesis functions as $S1PR_1$ in tumor progression.

2.2.2.3.4 $S1PR_4$ and $S1PR_5$

$S1PR_4$ is mainly expressed in the hematopoietic system. Its function inside the cells is not clear but $S1PR_4$ knockout mice showed defects in dendritic cell differentiation and cytokine secretion.[117]

$S1PR_5$ is predominately expressed in the brain and within natural killer cells. Even though the function is under investigation, recent research suggests $S1PR_5$ could inhibit migration and proliferation of esophageal cancer cells.[118]

In summary, $S1PR_{1\text{-}5}$ play crucial roles in various physiological and pathophysiological processes in human. For breast cancer, it is usually associated with the functions of $S1PR_{1\text{-}3}$. To get a better glimpse of whether $S1PR_{1\text{-}3}$ indeed affects breast cancer patient's survival, we extracted the Kaplan-Meier plots for survival versus mRNA expression level for $S1PR_{1\text{-}3}$ from patients' data deposited at the Human Protein Atlas (HPA) database (Figure 7).[119] Surprisingly, the expression of $S1PR_1$ does not exhibit any effect on the predicted patient survival probability

for breast cancer patients; whereas both $S1PR_2$ and $S1PR_3$ are favorable prognostic factors for breast cancer patient's survival. As shown above, activation of $S1PR_1$ can lead to cancer growth in both *in vitro* and *in vivo* experiments. Furthermore, $S1PR_1$ antagonists such as FTY720, were shown to be able to inhibit tumor angiogenesis *in vivo* and enhance therapeutic efficacy of doxorubicin.[18,120] Therefore, blocking $S1PR_1$ with its antibody, associated with the exogenous administration of S1P, might lead to activation of S1P-$S1PR_2$ and S1P-$S1PR_3$ pathways, which might be a favorable option for better treatment outcome for breast cancer.

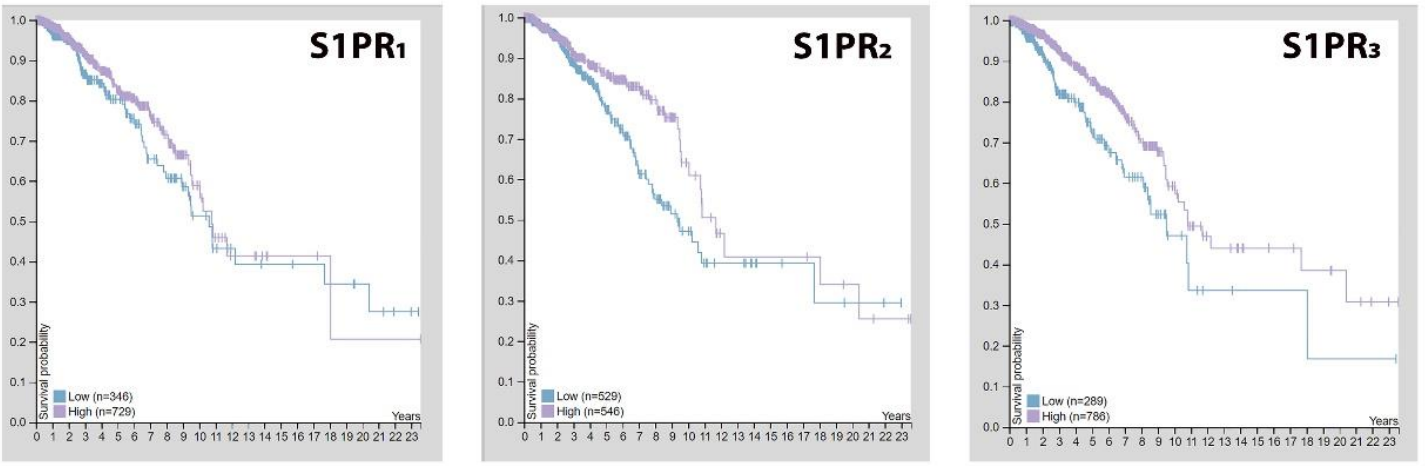


Figure 7. Kaplan-Meier plot of patient survival probability versus mRNA expression level for $S1PR_{1-3}$ based on breast cancer patients' data deposited at the Human Protein Atlas database.[119] URL for the images: https://www.proteinatlas.org/ENSG00000170989-S1PR1/pathology/tissue/breast+cancer, https://www.proteinatlas.org/ENSG00000267534-S1PR2/pathology/tissue/breast+cancer, https://www.proteinatlas.org/ENSG00000213694-S1PR3/pathology/tissue/breast+cancer. Images available from v18.proteinatlas.org.

2.2.3 S1P intracellular functions

The intracellular function of S1P is not fully understood, but the evidence thus far suggests S1P is an intracellular messenger (Figure 8). Recent studies suggest that intracellular S1P produced by nuclear SK2 can directly bind to and inhibit histone acetylation 1/2 (HDAC1/2) which removes acetyl groups from histones to epigenetically regulate target genes and reduce their generation, such as IL-6 and TNF-α in mouse lungs.[121–123] S1P generated by SK1/2 can regulate amyloid-β (Aβ) by inhibiting BACE1 in Alzheimer's disease (AD).[124] Intracellular S1P

produced by SK1 was found to be a cofactor for TNF receptor-associated factor 2 (TRAF2), which can lead to polyubiquitination of receptor interacting protein 1 (RIP1) and activation of the NF-κB pathway to inhibit apoptosis.[125] Besides, in mitochondria, S1P with prohibitin 2 (PHB2), a protein localized on the inner mitochondrial membrane, shows vital roles in mitochondrial respiration.[126]

In addition, S1PRs have been shown to localize inside the nucleus.[4] It has been demonstrated that $S1PR_2$ exists inside the nucleus and nuclear $S1PR_2$ inhibits the growth of estrogen receptor-negative breast cancer cells.[127] Furthermore, intracellular S1P produced by SK2 can bind to $S1PR_4$, causing nuclear translocation of $S1PR_2$, thereby promoting cell growth.[127] As well, SK2-derived S1P mediates epidermal growth factor-induced cancer cell invasion *via* the activation of intracellular $S1PR_2$.[128] Recent studies demonstrate that S1P produced by SK2 can act as an acute ezrin-radixin-moesin (ERM) activator, which can facilitate signal transduction between the extracellular matrix and the cytosol,[128] through its action on $S1PR_2$.[128]

Although much progress has been made for the intrinsic functions on improving cell survival, the anti-tumorigenesis effect of S1P has been shown to relate to S1P intrinsic functions.[129] For example, Wang *et al.* found that exogenous administration of S1P inhibits the chemotactic motility of human breast cancer MCF-7 and MDA-MB-231.[129] Through using caged S1P that could bypass cell surface receptors, it was further demonstrated that S1P inhibition of the motility was independent of the receptor and was associated with the intracellular action of S1P.[129]

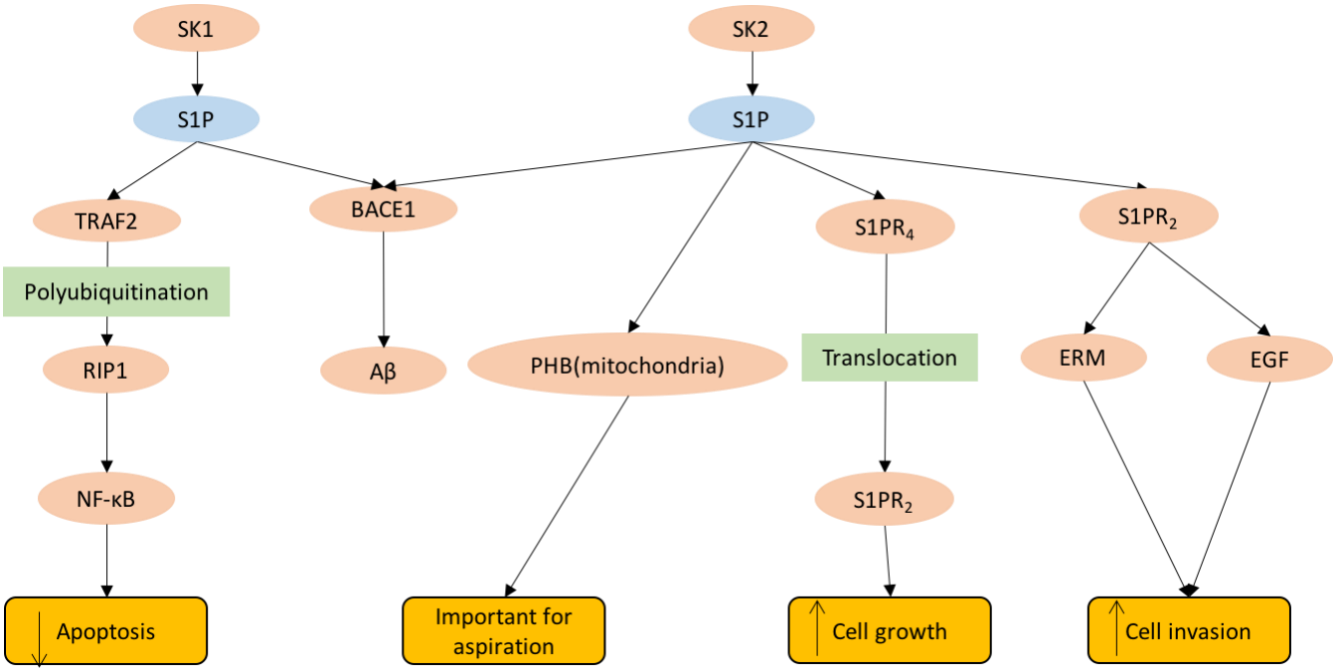


Figure 8. Functions of intracellular S1P that have been discovered so far. S1P produced by SK1 inside the cells can inhibit apoptosis by activating NF-κB protein. S1P synthesized by SK2 can bind to PHB2 on the inner mitochondrial membrane and help maintain mitochondrial function on aspiration. It can also induce cell growth and cell invasion *via* $S1PR_2$ inside the cells.

2.2.4 S1P and breast cancer

Evidence suggests SK1/2 and S1P produced by SK1 could work as tumor-promoting molecules, and their elevation has been observed in different types of cancer and tumor tissues. For breast cancer, compared with normal breast tissue, expression of SK1 was found to be elevated in breast cancer cells.[61] Poor prognosis and promotion of metastasis are related to higher SK1 expression.[61] For example, in MCF-7 breast cancer cells, overexpression of SK1 enhanced estradiol-dependent tumor formation.[130] Also, SK1 is a key regulator of breast cancer progression as SK1 was required for EGF-directed motility.[80] EGF and EGFR downstream signaling has an essential role in the progression, invasion, and maintenance of malignant phenotype of breast cancer.[80] Hence, down-regulating SK1 could reduce EGF- and serum-stimulated growth and enhance sensitivity to doxorubicin, a potent chemotherapeutic agent.[80] For SK2, even though its connection with cancer is unclear, its down-regulation in MCF-7 decreases G2-M arrest and enhances apoptosis induced by doxorubicin dramatically.[84] In

mouse tumor model, SK2-deficient breast cancer cells impair its growth and indicate its function on cell growth.[126,131]

S1P and SK2 also work as anti-tumorigenesis molecules. As mentioned above, SK2 was found to suppress growth and markedly enhance apoptosis that was independent of S1PRs.[78] Intracellularly, SK2-produced S1P could act as an endogenous HDAC inhibitor,[132] suggesting a more sophisticated role of SK2 in cancer progression based on different epigenetic regulation contexts among different cell types.

It is speculated that the anti-tumor function of exogenous S1P on breast cancer is due to its intracellular role. Other than studies by Wang et al.[129], our previous studies showed that S1P could selectively induce cell apoptosis of breast cancer cells at a concentration higher than 1μM.[133] At these concentrations, both $S1PR_1$ and $S1PR_2$ were expected to be blocked by ligand saturation. S1P would accumulate inside the cells, induce apoptosis, and inhibit migration *via* its intracellular functions.

2.2.5 S1P and chemo-resistance

Although progress has been made in diagnosis and treatment of breast cancer in the past several decades, drug resistance and toxicity of therapy remain the primary causes for failure treatment of breast cancer.[21,40,47,53] It has been shown that endogenous level of ceramides and S1P are essential to chemotherapy resistance.[134] Alteration of ceramide accumulation can cause cancer cells to develop resistance towards chemotherapy.[61] Knockdown of glucosylceramide synthase (GCS), which synthesizes glucosylceramide (GlcCer) from ceramide, inhibits MDR1 gene expression to reduce P-glycoprotein (P-gp) and reverse drug resistance.[61] During the development of drug resistance, GlcCer accumulation was also observed.[135,136] In cisplatin-resistant melanoma cells, the combination of cisplatin with FTY720, an agonist to S1PRs except for $S1PR_2$, can reduce cell proliferation and induce cell death.[137] At the same time, the combination of FTY720 and cisplatin also decrease PI3K, Akt, and EGFR expression levels, critical proteins for cell survival.[137,138] This experiment, once again, demonstrates the importance of endogenous S1P in cancer cells that have developed chemotherapy resistance.[137,138]

In addition, overexpression of sphingosine kinases, which are essential for ceramide conversion into S1P, is related to chemo-resistance in many cancers. [134,139,140] For example, in breast cancer MCF-7 cells, overexpression of SK1 results in increased resistance to doxorubicin, tamoxifen and necrosis factor.[134,139] In non-small cell lung carcinoma cells, overexpression of SK2 has been related to gefitinib resistance.[140]

3 Hypothesis and Objectives

Sphingosine-1-phosphate (S1P), a pleiotropic lysophospholipid mediator, can regulate S1P receptor (S1PR)-dependent or S1PR-independent cell proliferation, migration, survival, and differentiation.[91,95] Previous studies from our laboratory have shown that exogenously administered S1P can cause cell death in the breast cancer cell lines, MDA-MB-231 and MCF-7, in a concentration-dependent way.[70,141] Based on the pro-proliferation functions of $S1PR_1$ on the cell surface, we assume that the reason for low concentrations of S1P to induce cell proliferation is due to the activation of this receptor. Thus, blocking $S1PR_1$ signaling *via* ligand saturation would be effective in decreasing cell proliferation.

Moreover, results in our lab have also shown that exogenous S1P enhances the cytotoxic effect of docetaxel, doxorubicin, and cyclophosphamide towards MDA-MB-231 and MDA-MB-361 cells.[133] Based on the evidence, we hypothesize that S1P or $S1PR_1$ antibody can enhance the cytotoxic effect of carboplatin towards MCF-7, SK-BR-3, and MDA-MB-231 cells.

3.1 Hypothesis

I. $S1PR_1$ antibody enhances S1P cytotoxicity against breast cancer cells by blocking $S1PR_1$ on the cell surface.

II. S1P and $S1PR_1$ antibody in combination can enhance the cytotoxic effect of anti-cancer drug, carboplatin.

3.2 Objectives

I. Assess the cytotoxic effects of gradient concentrations of $S1PR_1$ antibody alone and in combination with exogenous S1P against MCF7, SK-BR-3 and MDA-MB-231 cell lines.

II. Assess the cytotoxic effect of S1P, $S1PR_1$ antibody and carboplatin in pairs on breast cancer cell lines MCF-7, SK-BR-3, and MDA-MB-231.

4 Methods

4.1 Materials

Sphingosine-1-phosphate, carboplatin, MTT(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) were purchased from Sigma-Aldrich (Oakville, ON, Canada). $S1PR_1$ antibody was purchased from Abcam (Cambridge, MA, US). Penicillin solution was purchased from Sigma Life Science (Kansas City, MO, USA). Leibovitz's L-15 media, Eagle's Minimum Essential media, McCoy's 5a Medium Modified and Fetal Bovine Serum (FBS) were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). 0.25 % Trypsin EDTA, bovine insulin and human epidermal growth factor were purchased from Sigma Life Science. CellTox™ Green Cytotoxicity Assay kit was from Promega (Madison, WI, USA). Cell Cytotoxicity Assay kit-colorimetric from Abcam (Cambridge, MA, USA).

Cell lines: MDA-MB 231 cells (source: human breast adenocarcinoma, derived from pleural effusion), MCF-7 cells (source: human breast adenocarcinoma, derived from pleural effusion) and the SK-BR-3 cells (source: human mammary gland; breast) were purchased from ATCC.

4.2 Cell culture

According to the ATCC protocol (Table 4), MCF-7, SK-BR-3, and MDA-MB-231 cell lines were cultured in 75 cm^2 flasks. MDA-MB-231 cells were cultured in Leibovitz's L-15 medium supplemented with 10% FBS and 1% penicillin under 100% air without CO_2. MCF-7 cells were cultured in Eagle's Minimum Essential Medium supplied with 0.01 mg/mL bovine insulin, 10% FBS and 1% penicillin. SK-BR-3 cells were cultured in McCoy's 5a Medium Modified with 10% FBS and 1% penicillin. MCF-7 and SK-BR-3 cells were cultured in 95% air and 5% CO_2. Cell culture medium was changed every 2-3 days.

Table 3: The cell culture conditions for different breast cancer cell lines used in the experiments

Cell line	Receptor Expression			Source	Culture Medium	Incubation condition	Molecular Classification
	ER	PR	HER2				
MCF-7	√	√	—	Pleural Effusion	MEM, 10%FBS, 0.01mg/mL insulin	5%CO_2, 37℃	Luminal A
SK-BR-3	—	—	√	Pleural Effusion	McCoy 5a medium, 10%FBS	5%CO_2, 37℃	HER2
MDA-MD-231	—	—	—	Pleural Effusion	L-15, 10%FBS	No CO_2, 37℃	Normal breast-like
MEM: Eagle's minimum Essential Medium; L-15: Leibovitz's L-15 medium							

4.3 Drug preparation

Carboplatin powder was diluted with ddH_2O to get a stock solution 27 mM and sterilized by pressing through a 0.20 µm filter. Final carboplatin dilutions of 4.2 – 1,080 µM were used in the combination experiments.

In assessing the IC_{50} value of carboplatin in three breast cancer cell lines, carboplatin powder was diluted with corresponding growth medium. Final carboplatin dilution of 1.95 -1,000 µg/mL for MCF-7 cell line, 0.98 -1,000 µg/mL for SK-BR-3 cell line, and 7.8 – 2,000 µg/mL for MDA-MB-231 cell line. New solutions were made for each experiment.

To dissolve the S1P powder, methanol with 120 mg/mL polyethylene glycol (PEG) was prepared and S1P powder was diluted with methanol (120 mg/mL PEG) to obtain a stock solution of 2.64 mM. Final concentrations of 0.08 µM – 10 µM were made immediately before use.

4.4 Toxicity assay

4.4.1 Assess the toxic effect of $S1PR_1$ antibody

Depending on the particular cellular process of interest, different toxicity assays can be

used. In this project, two different toxicity assays were used – CellTox™ Green Cytotoxicity assay and MTT assay to assess the cytotoxicity of cells. The CellTox™ Green Cytotoxicity assay uses a proprietary asymmetric cyanine dye (CellTox™ Green Dye) which only binds to the dead cells' DNA. Therefore, the fluorescence signal is proportional to the cytotoxicity at 485/510 nm excitation source and 520 – 530 nm emission.[142] The MTT assay can assess cell viability. The principle of MTT assay is that the NADH, which is a coenzyme in all living cells, could reduce MTT to crystal and insoluble purple product – formazan. Therefore, the quantity of formazan is presumably directly proportional to the number of viable cells.

4.4.1.1 Using CellTox™ Green Cytotoxicity assay

To assess the cytotoxic effect of $S1PR_1$ antibody with CellTox™ Green Cytotoxicity assay kit, I seeded MCF-7 cells, MDA-MB-231 cells and SK-BR-3 cells at a density of 8,000 cells/well into 96-well plates. After 24 h, the medium was changed with medium containing 2 µL/mL green dye. Cells were treated with $S1PR_1$ antibody. After 12 h, 18 h, 24 h, 48 h, 72 h, a Biotek microplate reader was used to quantitatively measure the dye's fluorescence signal at 485/510nm excitation source and 520 – 530 nm emission. The percentage of cell cytotoxicity was calculated using the formula:

$$\%\text{Cell cytotoxicity} = \frac{Experiment - Negative\ control}{Maximum\ cell\ death - Negative\ control} \times 100\%$$

4.4.1.2 Using MTT assay

MCF-7 cells, MDA-MB-231 cells, and SK-BR-3 cells were seeded at a density of 8,000 cells/well into 96-well plates. After 24h, cells were treated with $S1PR_1$ antibody (16 – 4,000 ng/mL). After 24 h (only for SK-BR-3), 48 h, or 72 h, the medium was replaced to medium with MTT (0.5 mg/mL). After treatment with MTT, the absorbance was measured at 570 nm with Biotek microplate reader. The percentage of viable cells was calculated using the formula:

$$\%\text{Cell viability} = \frac{OD(sample) - OD(background)}{OD\ (vehicle\ control) - OD(background)} \times 100\%$$

4.4.2 Assess the IC_{50} values of carboplatin

The MTT assay was performed to determine the IC_{50} value for MCF-7, SK-BR-3, and MDA-MB-231 cell lines. In brief, cells were seeded at 8,000 cells/well in 96-well plates. After 24

h, media was removed and new media containing carboplatin (1.95 – 1,000 μg/mL for MCF-7 cell line, 0.98 – 1,000 μg/mL for SK-BR-3 cell line, and 7.8 – 2,000 μg/mL for MDA-MB-231 cell line) was added. After 48 h or 72 h, the medium was replaced with MTT (0.5 mg/mL). After treatment with MTT, the absorption was measured at 570 nm using a Biotek microplate reader. The percentage of viable cells was calculated using the formula:

$$\%\text{Cell viability} = \frac{OD(sample) - OD(background)}{OD\ (vehicle\ control) - OD(background)} \times 100\%$$

Concentration-response curves were drawn. The IC_{50} values were determined by using the four-parameter nonlinear regression equation with variable slope in GraphPad Prism (GraphPad Prism 6.0 software, La Jolla, CA).

4.4.3 Assess the cytotoxicity of S1P, S1PR$_1$ antibody, and carboplatin in pairs

MCF-7 cells, MDA-MB-231 cells and SK-BR-3 cells were seeded at a density of 8,000 cells/well into different 96-well plates. After 24 h, cells were treated with S1PR$_1$ antibody (80 or 4,000 ng/mL) combining with S1P (0.08 – 10 μM), S1P (0.08 – 10 μM) combined with carboplatin (4.2 – 1,080 μM), or carboplatin (4.2 – 1,080 μM) combined with S1PR$_1$ antibody (80 or 4,000 ng/mL). After 48h and 72h, the absorption was measured following the MTT protocol mentioned above.

4.5 Data analysis

Except for the experiments on determining the IC_{50} value of carboplatin, all results for toxicity assays were pooled and represented as mean values ± standard deviation. Data were presented as histograms. Two-way ANOVA with Sidak analysis was used to analyze the results. Significance was set at $P \leq 0.05$. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

5 Results and discussion

5.1 S1PR$_1$ antibody exhibited cytostatic in breast cancer cells

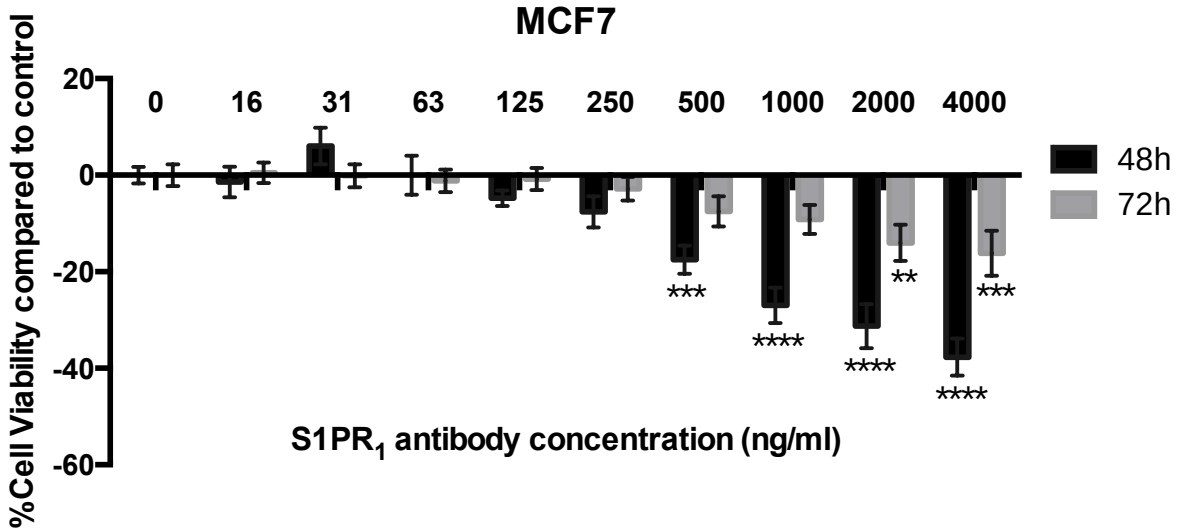
The antitumor effect of S1PR$_1$ antibody against breast cancer cells was evaluated in three frequently used human breast cancer cell lines, MCF-7, SK-BR-3, and MDA-MB-231, using both MTT assay and CellTox Green Cytotoxicity Assay. MCF-7 (luminal A subtype) represents breast cancer that is amenable to hormone therapy and is less invasive compared

with SK-BR-3 and MDA-MB-231 cells.[143] SK-BR-3 (HER2 subtype) cell line represents those that can respond to targeted therapy.[143] MDA-MB-231 (triple-negative subtype) cell line represents the breast cancer that is more biologically aggressive and often has a poor prognosis.[143] Although the binding affinity of this antibody to $S1PR_1$ is not reported, the manufacturer recommended a final concentration of 200 ng/mL - 2,000 ng/mL for Western Blot and 1,000 ng/mL – 4,000 ng/mL for Flow Cytometry studies. Therefore, we used concentration of 16 – 4,000 ng/mL in both assays.

$S1PR_1$ antibody alone showed cytostatic effect on the three human breast cancer cell lines in a concentration- and time-dependent manner, although the cell lines responded differently against $S1PR_1$ antibody and the response was mild. In MTT assay (Figure 9), $S1PR_1$ antibody decreased the cell viability at concentrations above 500 ng/mL in MCF-7 cells. Up to 40% reduction of cell viability was observed in MCF-7 after exposing to 4,000 ng/ml $S1PR_1$ antibody for 48 h. Comparing 48 h with 72 h exposure time for 500, 1,000, 2,000 and 4,000 ng/ml $S1PR_1$ antibody treatments, respectively, the cell viability was reduced more at 48 h. At the range of 16 – 4,000 ng/mL, the maximum effect of ~ 10% cell viability reduction was achieved at the concentration of 4,000 ng/mL after treating for 72 h in SK-BR-3 cells. Prolonged exposure did not enhance the effect of $S1PR_1$ antibody on MCF-7 cells and SK-BR-3 cells. Meanwhile, the cell growth of MDA-MB-231 cells was promoted by $S1PR_1$ antibody at low concentrations (16 - 500 ng/mL) while it was inhibited at high concentrations (1,000 - 4,000 ng/mL). At low concentrations (16 – 500 ng/ml) of $S1PR_1$ antibody, the percentage of living cells exhibited a bell-shaped dose-response relationship. The maximum promoting effect of cell growth (~ 10% increase in cell viability) was at 125 ng/mL $S1PR_1$ antibody for 72 h; whereas within the range of the given concentrations, the maximum inhibiting effect on cell growth (~ 27% decrease in cell viability) was at a concentration of 4,000 ng/mL $S1PR_1$ antibody for 72 h. Collectively, only at high concentrations (above 1,000 ng/mL), the $S1PR_1$ antibody achieves robust cytostatic effect on these three cell lines, which is consistent with literature evidence.[144] As cell viability was reduced after exposing to $S1PR_1$ antibody, we conclude that the commercial $S1PR_1$ antibody we bought is functionally effective.

As shown in Figure 10, $S1PR_1$ antibody did not cause a cytotoxic effect in SK-BR-3 cells

as examined with the CellTox™ Green Cytotoxicity assay while only ~ 10% cytotoxicity was observed in MDA-MB-231 cells at the concentration of 4,000 ng/mL $S1PR_1$ antibody after 72 h. We speculated that the cytotoxicity might be caused by the differences of $S1PR_1$ expression and internalization between the two cell lines. However, we could not completely rule out the possibility that a different antigen possesses a fragment of sequence highly homologous to the epitope used in producing the $S1PR_1$ antibody and binds $S1PR_1$ antibody in the MDA-MB-231 cells. In addition, a bell-shape dose response relation between $S1PR_1$ antibody at low concentration and percentage living cells was observed in MDA-MB-231 cells. We speculate that this was caused by cellular production of S1P when $S1PR_1$ was not completely blocked by its antibody. A similar bell-shape dose-response was also observed in MCF-7 cells at low concentrations of S1P (0.03 – 2 μM) in the previous study.[141] As mentioned above, the MTT assay, where MTT can be reduced to formazan by coenzyme in living cells, is used to measure the cell viability. The CellTox™ Green Cytotoxicity assay, where the green dye can only bind to DNA of dead cells, is used to estimate the cell death. Therefore, due to the experimental results observed from both detection methods, we consider that the effect on cell growth of $S1PR_1$ antibody on the MCF-7, SK-BR-3 and MDA-MB-231 cells is likely *via* the inhibition of cell growth (cytostatic effect).



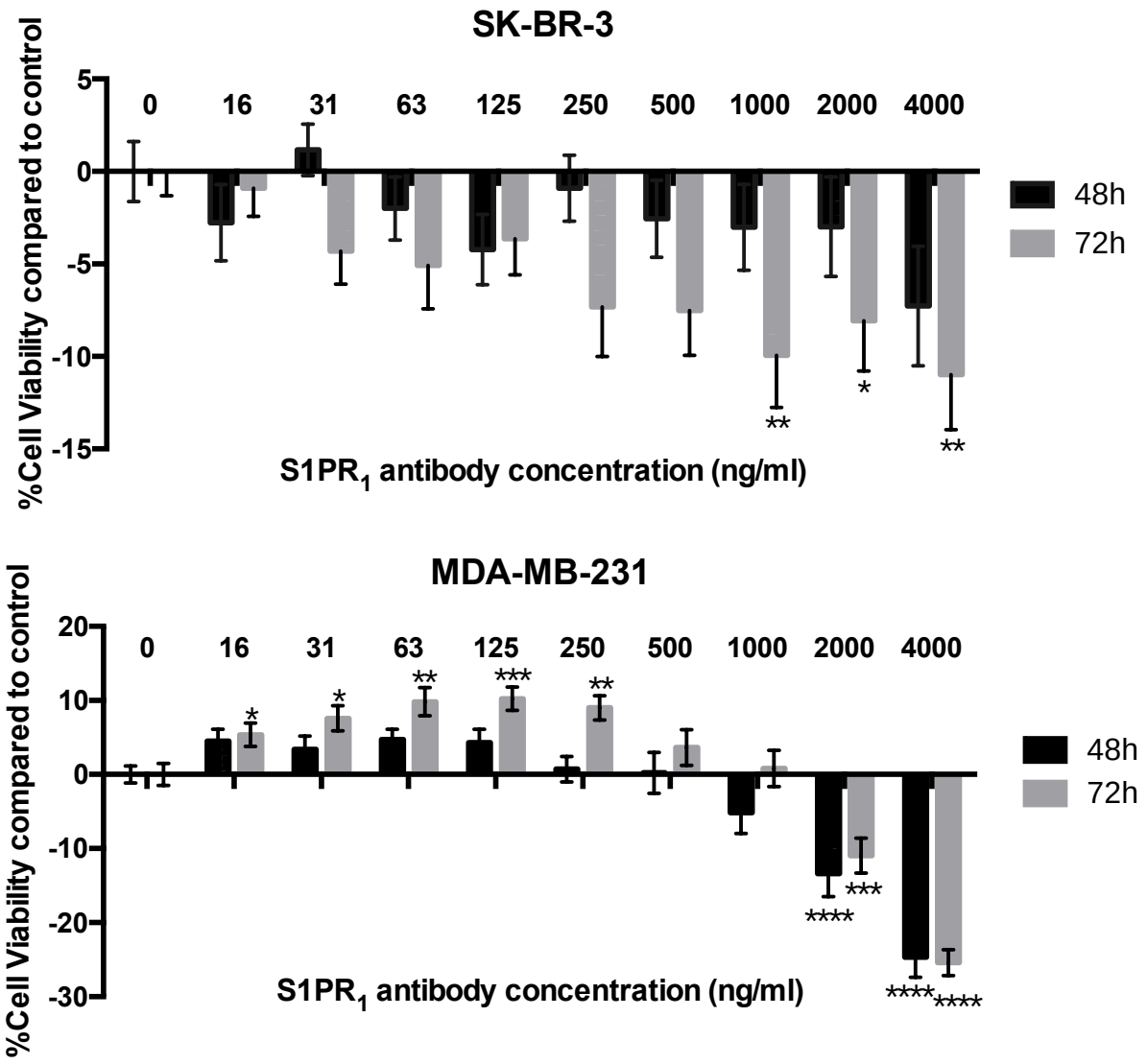


Figure 9. Toxic effect of $S1PR_1$ antibody against MCF-7, SK-BR-3, and MDA-MB-231 cells. Cells were treated with different concentrations of $S1PR_1$ antibody (16 – 4000 ng/mL) for 48 h and 72 h. PBS was used as vehicle control. MTT Assay was performed to determine the percent cell viability. Results are presented from triplicate assays on three different occasions. Two-way ANOVA with Sidak analysis was used to analyze the significance between cells treated with different concentration of $S1PR_1$ antibody and cells treated with vehicle control. The data are reported as mean ± SD (N=3), significance was set as $P \leq 0.05$. Note: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$.

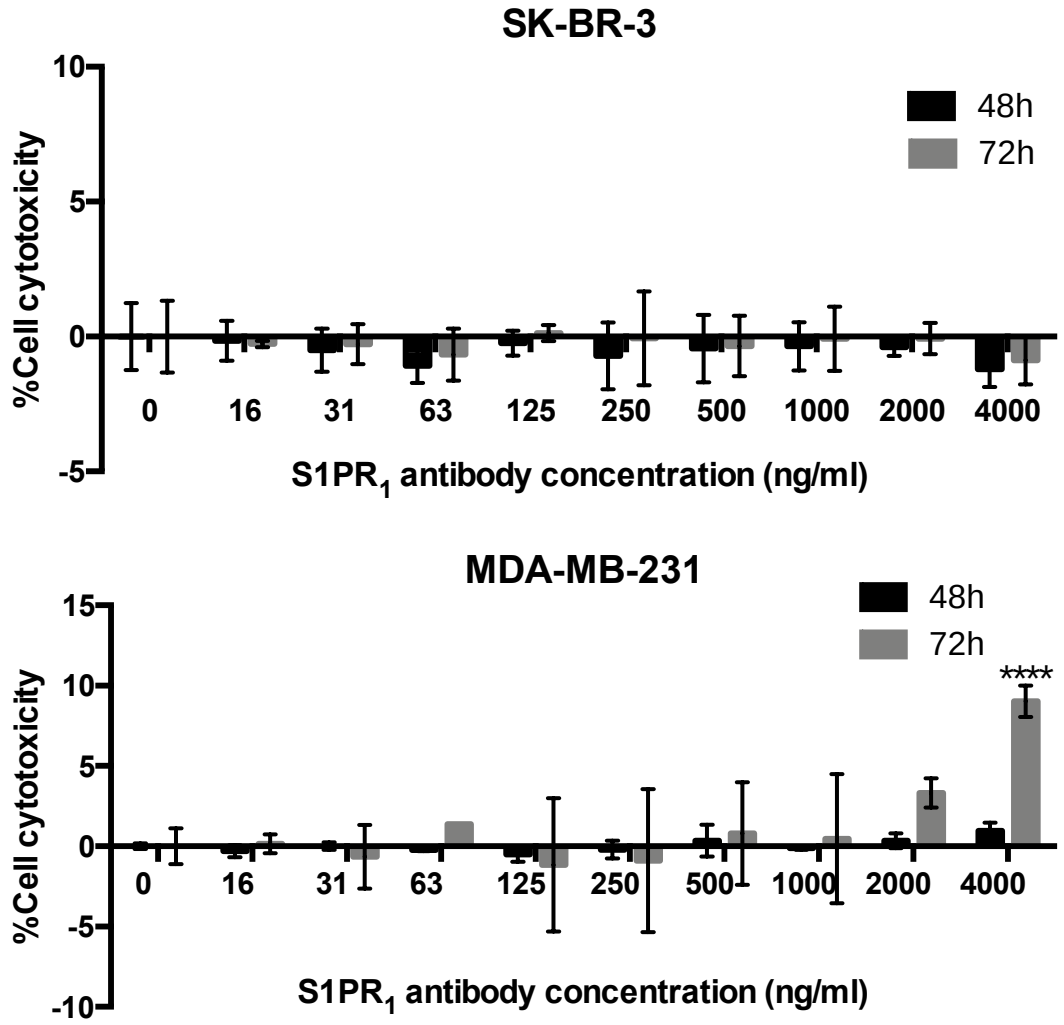


Figure 10. The cytotoxic effect of $S1PR_1$ antibody on MDA-MB-231 and SK-BR-3 cells evaluated by CellTox™ Green Cytotoxicity Assay. Cells were exposed to $S1PR_1$ antibody (0-4000 ng/ml) for 48 h and 72 h. PBS was used as vehicle control. Results are presented from duplicate replications on two different occasions. Two-way ANOVA with Sidak analysis was used to analyze the significance between cells treated with different concentration of $S1PR_1$ antibody and cells treated with vehicle control. The data are reported as mean ± SD (N=2), significance was set as $P \leq 0.05$. Note: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$.

Taken together, the results indicate that these three breast cancer cell lines react differently to $S1PR_1$ antibody. One reason might be gene expression differences within these three cell lines. As shown in Figure 11, different subtypes of breast cancer cells have different cellular genotypes. For example, *phosphatase and tensin homolog (PTEN)*, which is a tumor

suppressor gene that prevents cells from dividing uncontrollably, exists in wild-type in these three cell lines. *Estrogen receptor (ER)*, which acts as a transcription factor after binding with estrogen, is overexpressed in MCF-7 cells while is normally expressed in both SK-BR-3 and MDA-MB-231 cells. *PIK3CA*, which encodes PI3K protein, is mutated in MCF-7 cells but normal in SK-BR-3 and MDA-MB-231 cells. *Human epidermal growth factor receptor 2* (*HER2* or *ERBB2*), an oncogene that plays a vital role in certain types of cancer progression, is overexpressed in SK-BR-3 cells, but exists in wild-type in MCF-7 and MDA-MB-231 cells. Furthermore, protein expression of the genes mentioned above, are the downstream reactors of $S1PR_1$. Thus, MCF-7, SK-BR-3, and MDA-MB-231 reacted differently to the exposure of $S1PR_1$ antibody. Moreover, because of the genotype difference, the metabolic rates of the $S1PR_1$ antibody in these two cell lines might be different.

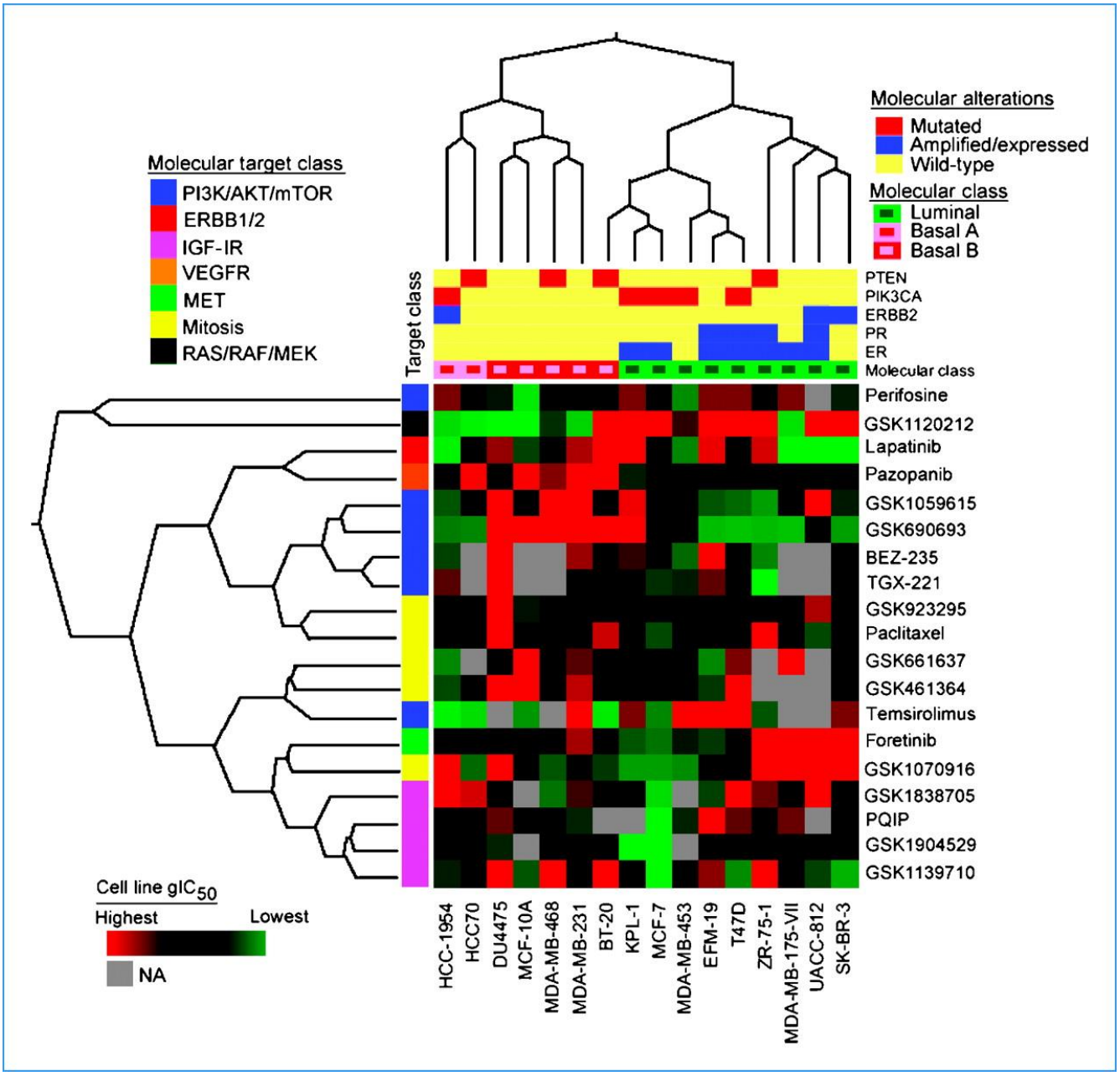


Figure 11. The clustering results of gIC_{50} values of 19 compounds (rows) on 16 breast cancer cell lines (columns) (P = 0.0009, Fisher's exact test). It shows that cellular genotype is a strong driver for different breast cancer cells reacting to compounds. Green and red cells represent cell lines with lower and higher gIC_{50} values, respectively, whereas grey cells indicate missing data.[145] Permission to reproduce this is granted by American Association for Cancer Research.

The second reason for the different responses of the three cell lines to $S1PR_1$ antibody is that the expression level of $S1PR_1$ is different in these cell lines. The expression levels of $S1PR_1$ in breast cancer tissues and breast cancer cells are ambiguous at present time; with results varying dramatically from different researchers. Information from The Human Protein Atlas database (HPA) show that the expression level of $S1PR_1$ in breast cancer tissues is downregulated compared to normal breast tissues. As shown in Table 4, the RNA-seq data of normal breast tissues was generated by the Genotype-Tissue Expression project from 214 tissue samples. The data were presented as average RPKM (reads per kilobase per million mapped reads). The RNA-seq data of breast cancer tissues were generated by The Cancer Genome Atlas from 1075 tissue samples. The data were presented as average FPKM (number Fragments per Kilobase of exon Million reads). Because RPKM is calculated from single-end RNA-seq results and FPKM is calculated from the paired-end RNA-seq, FPKM is equal to half value of RPKM. Therefore, we can see that the expression level of $S1PR_2$ and $S1PR_3$ did not change much in breast cancer tissues compared to normal breast tissues, $S1PR_1$ reduced tremendously in breast cancer tissues. In addition, from The Human Protein Atlas, the expression level of $S1PR_1$ in MCF-7 and SK-BR-3 cells was downregulated and that the TPM (transcripts per kilobase million) values of $S1PR_1$ in these two cell lines were closed to zero. However, as shown in Figure 12, another study shows that the expression of $S1PR_1$ was upregulated on 25 patient-derived breast cancer tissues and four breast cancer cell lines (MDA-MB-231, MCF-7, T-47D and BT-474) compared with normal breast tissues and breast cells, respetively.[146] From the results in this study, it is implied that the relative $S1PR_1$ expression level was higher in MDA-MB-231 cells than in MCF-7.[146] This may be the reason that in my experiment, exposed to 500 ng/ml $S1PR_1$ antibody for 48 h can cause about 20% cell viability reduction in MCF-7 cells while in MDA-MB-231 cells, only 15% cell viability reduction was observed after 48 h exposing to 2,000 ng/mL $S1PR_1$ antibody. In order to further demonstrate the relationship between the expression level of $S1PR_1$ antibody in breast cancer cells and the antitumor effect of $S1PR_1$ antibody, the expression status of $S1PR_1$ in different breast cancer cell lines should be further tested.

Table 4. The expression levels of S1PRs on normal breast and breast cancer tissues

	Normal breast tissues	Breast cancer tissues
	Average RPKM	Average FPKM
$S1PR_1$	67.7	8.0
$S1PR_2$	2.4	4.3
$S1PR_3$	2.9	6.2

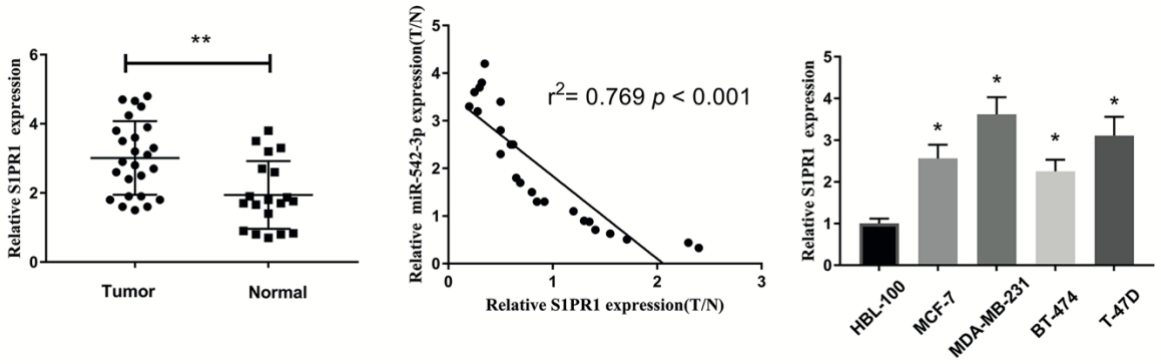


Figure 12. The expression level of $S1PR_1$ was detected by qRT-PCR. Expression of $S1PR_1$ was upregulated in breast cancer tissues samples from 25 patients compared with normal breast tissues, and in breast cancer cells (MDA-MB-231, MCF-7, T-47D and BT-474) compared with normal breast cells (HBL-100). *, $p \leq 0.05$; **, $p \leq 0.01$.[146] Permission to reproduce this is granted by Medical and Pharmacology Sciences.

In fact, many $S1PR_1$ agonists and antagonists have been developed in recent years for cancer treatment. For example, $S1PR_1$ antagonists were shown to be able to inhibit tumor angiogenesis *in vivo* and enhance therapeutic efficacy of doxorubicin.[18,120] But adverse effects always appear during the treatments. For example, fingolimod (FTY720), an antagonist of all S1PRs except $S1PR_2$, has been clinically approved for the treatment of relapsing and remitting multiple sclerosis.[93] Use of fingolimod has clinical adverse effects – significant transient bradycardia because of its effect on cardiac $S1PR_3$.[93] In addition, FTY720P, the active metabolite of FTY720, is a functional antagonist through promoting internalization of $S1PR_1$. However, persistent signaling, function as agonist of $S1PR_1$, was observed after short time exposure to FTY720P.[93,147] Therefore, monoclonal $S1PR_1$ antibody is used in my experiment to avoid the confusing functions of FTY720P. Using $S1PR_1$ antibody may be associated with

fewer side effects compared with current agonists and antagonists of S1PRs such as fingolimod.

Results show that $S1PR_1$ antibody caused cytostasis in the three breast cancer cell lines but due to the diverse functions of $S1PR_1$, the mechanism of the inhibition effect of $S1PR_1$ antibody on cell growth in breast cancer cells is still unrevealed. Blocking $S1PR_1$ on the cell surface with its antibody can lead to inhibition of PI3K, PLC and Ras GTPase protein and their downstream pathways, which involve numerous proteins and molecules. For example, blocking $S1PR_1$ with its antibody can lead to inhibition of ERK protein and upregulation of pro-apoptosis protein Bim, finally inducing cell apoptosis by activating caspase 3 protein.[148] In addition, it is possible that after blocking $S1PR_1$ with its antibody on the cell surface, S1P produced by the cells themselves can bind to $S1PR_2$ to reduce cell viability or through the influx into cells and act as a second messenger to elude downstream reactions.

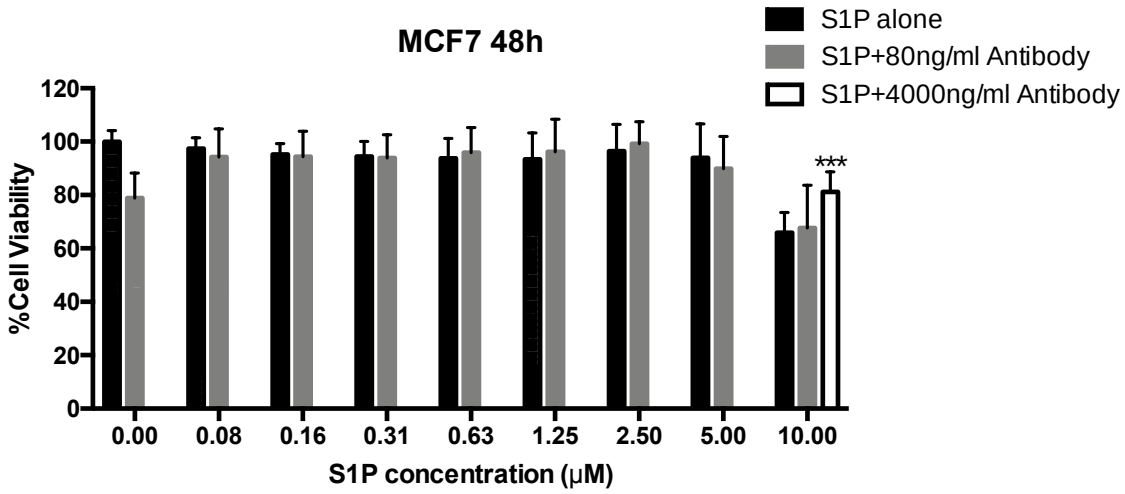
5.2 S1P activity was not enhanced by $S1PR_1$ antibody in breast cancer cells

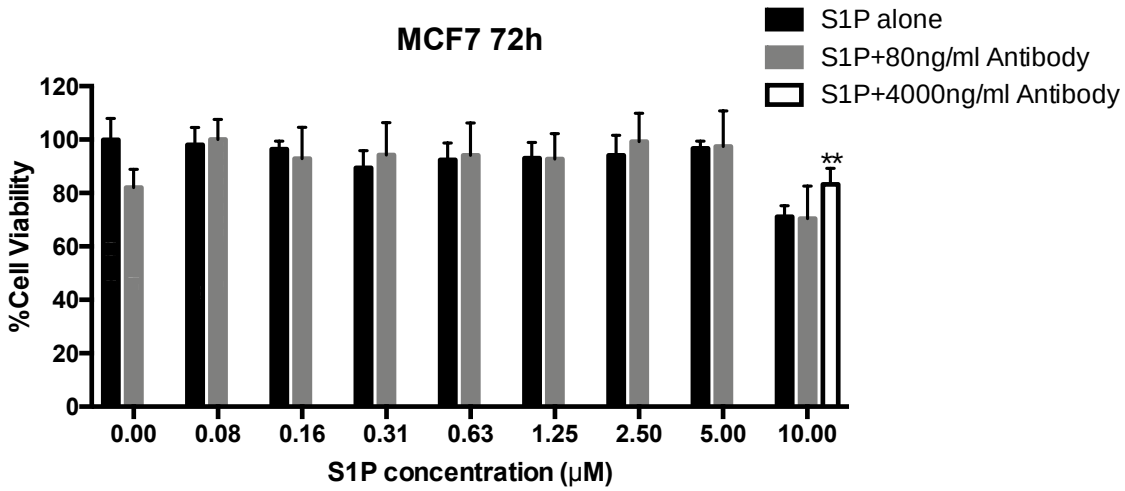
To assess the cytotoxic effects of S1P combined with $S1PR_1$ antibody in MCF-7, SK-BR-3, and MDA-MB-231 cells, MTT assay and CellTox™ Green Cytotoxicity assay were performed. First, MTT assay was carried out at fixed $S1PR_1$ antibody concentration of 80 ng/mL with different concentrations of S1P in the three aforementioned cell lines. As shown in Figure 13, combination with 80 ng/mL $S1PR_1$ antibody did not enhance the effect of S1P on cell viability in MCF-7 cells. Co-administration with 4,000 ng/mL $S1PR_1$ antibody increased cell viability about 10% when comparing with 48-hour treatment of S1P alone at 10 μM, so as with 72-hour treatment. Compared with cells treated with 10 μM of S1P, no significant reduction on cell viability was observed in SK-BR-3 cells treated with 4,000 ng/mL $S1PR_1$ antibody and 10 μM of S1P for 24 h. However, co-administration of 4,000 ng/mL $S1PR_1$ antibody decreased the cell viability by almost 20% ($p \leq 0.0001$) after 48 h treatment comparing with 10 μM of S1P alone. At 72 h of treatment, the co-administration of $S1PR_1$ antibody reduced the cell viability by approximately 10%, but this decrease is not statistically significant. For the MDA-MB-231 cell line, S1P alone and combined with $S1PR_1$ antibody did not show any significant difference with each other. Subsequently, we evaluated the cytotoxic effect of the combination of S1P at two fixed concentrations (1 and 10 μM) with $S1PR_1$ antibody at various concentrations in the three cell lines using the CellTox™ Green Cytotoxicity Assay. $S1PR_1$ antibody combined with 10 μM

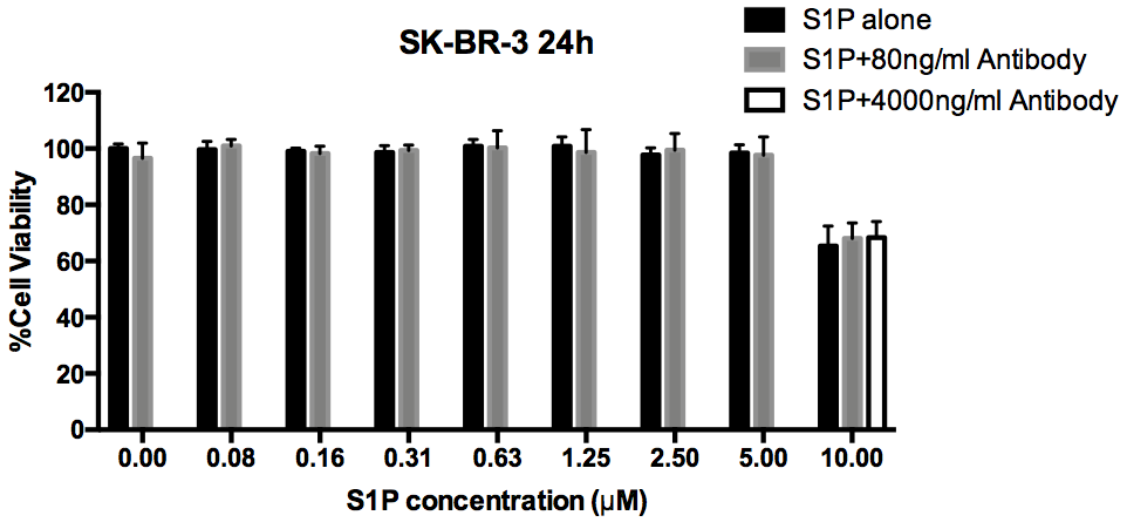
S1P were used to evaluate whether there could be any synergistic/additive effect. 10 μM of S1P was chosen based on the previous studies in our lab. Results from our lab showed that S1P selectively induced cell apoptosis in breast cancer MCF-7 cells and exhibited synergistic effect with chemotherapy drugs docetaxel, doxorubicin and cyclophosphamide in breast cancer cell lines MCF-7 and MDA-MB-361 at a concentration higher than 1 μM, especially at 10 μM.[70,141] As shown in Figures 14, 15 and 16, no significant differences in cell death were observed in MCF-7, SK-BR-3 and MDA-MB-231 cells as compared to negative control when S1P and $S1PR_1$ antibody were co-administered. In conclusion, these results suggest that $S1PR_1$ antibody did not enhance the cytotoxic effect of S1P towards the MCF-7 and MDA-MB-231 cells. For the SK-BR-3 cells, $S1PR_1$ antibody showed marginal enhancement on the cytotoxic effects of S1P.

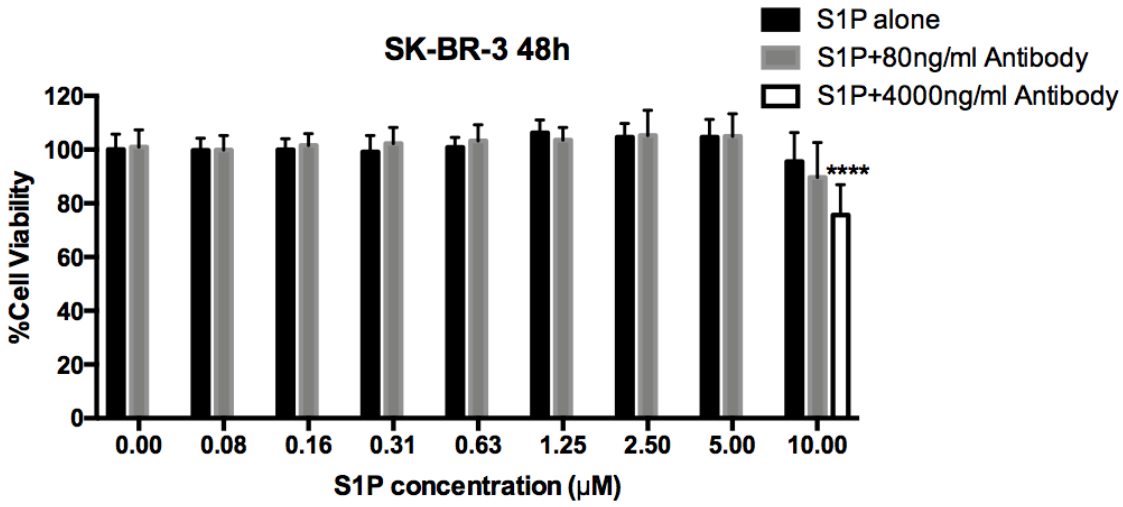
We hypothesized that high concentration of S1P (10 μM) could block $S1PR_s$ on the cell surface and cause cell death through the intracellular function of S1P. $S1PR_1$ antibody may prolong the cytotoxic effect of S1P in SK-BR-3 cells by preventing the binding of S1P to the $S1PR_1$ on the cell surface even after it is degraded to lower concentrations. However, further exploration is still needed to confirm this hypothesis.

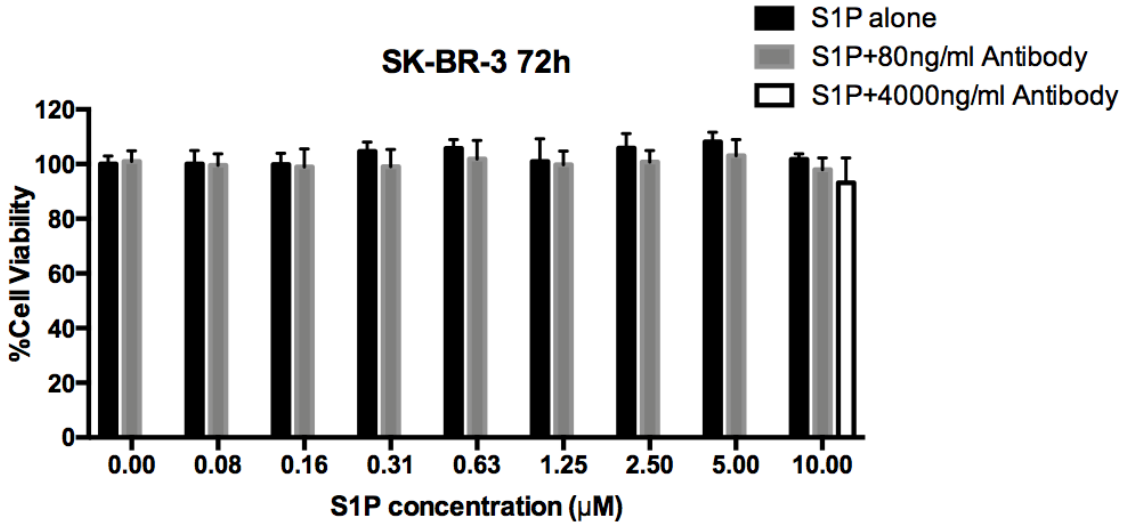
The combination of 10 μM S1P and 4,000 ng/mL $S1PR_1$ antibody affects the cell growth of MCF-7, SK-BR-3 and MDA-MB-231 cells differently. One reason for this is the differences of gene expression in these three cell lines, similar as the cause of cell growth effect for $S1PR_1$ antibody. Another reason might be the different degradation rates of S1P in these three cell lines, but the exact values are still unclear. In this study, cell viability reduction was observed when MCF-7 and MDA-MB-231 cells were treated with 10 μM of S1P for both 48 h and 72 h, while no cytotoxicity was detected in SK-BR-3 cells. A previous study of our lab showed that 1 μM S1P was degraded within 24 h in SK-BR-3 cells. It is possible that the degradation rate of S1P in the MCF-7 and MDA-MB-231 is slower as compared with that in the SK-BR-3 cells.

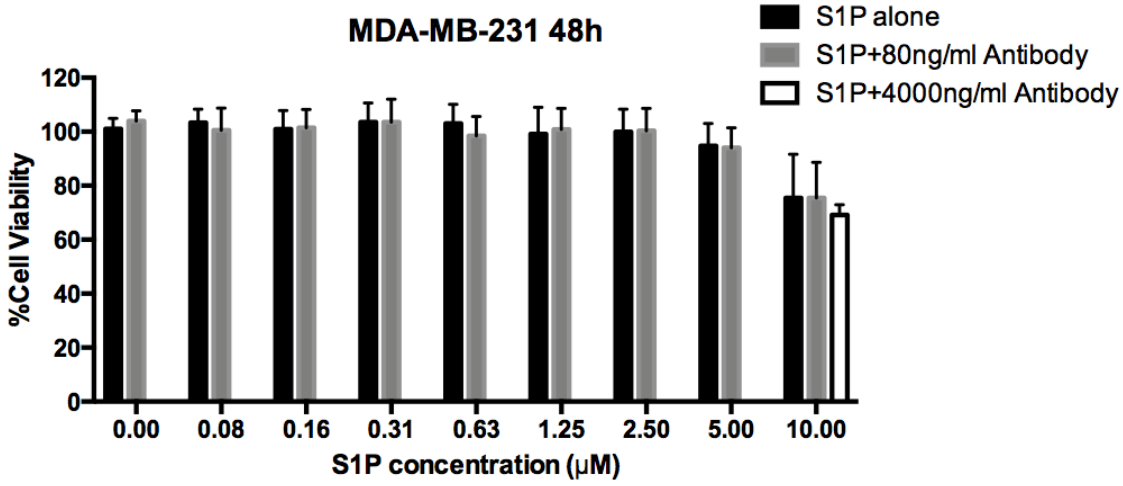

MCF7 48h
S1P alone
S1P+80ng/ml Antibody
S1P+4000ng/ml Antibody
%Cell Viability
120
100
80
60
40
20
0
***
0.00
0.08
0.16
0.31
0.63
1.25
2.50
5.00
10.00
S1P concentration (µM)


MCF7 72h
S1P alone
S1P+80ng/ml Antibody
S1P+4000ng/ml Antibody
%Cell Viability
120
100
80
60
40
20
0
**
0.00
0.08
0.16
0.31
0.63
1.25
2.50
5.00
10.00
S1P concentration (µM)


SK-BR-3 24h
S1P alone
S1P+80ng/ml Antibody
S1P+4000ng/ml Antibody
%Cell Viability
120
100
80
60
40
20
0
0.00
0.08
0.16
0.31
0.63
1.25
2.50
5.00
10.00
S1P concentration (µM)


SK-BR-3 48h
S1P alone
S1P+80ng/ml Antibody
S1P+4000ng/ml Antibody
%Cell Viability
120
100
80
60
40
20
0
****
0.00
0.08
0.16
0.31
0.63
1.25
2.50
5.00
10.00
S1P concentration (μM)


SK-BR-3 72h
S1P alone
S1P+80ng/ml Antibody
S1P+4000ng/ml Antibody
%Cell Viability
120
100
80
60
40
20
0
0.00
0.08
0.16
0.31
0.63
1.25
2.50
5.00
10.00
S1P concentration (μM)


MDA-MB-231 48h
S1P alone
S1P+80ng/ml Antibody
S1P+4000ng/ml Antibody
%Cell Viability
120
100
80
60
40
20
0
0.00
0.08
0.16
0.31
0.63
1.25
2.50
5.00
10.00
S1P concentration (μM)

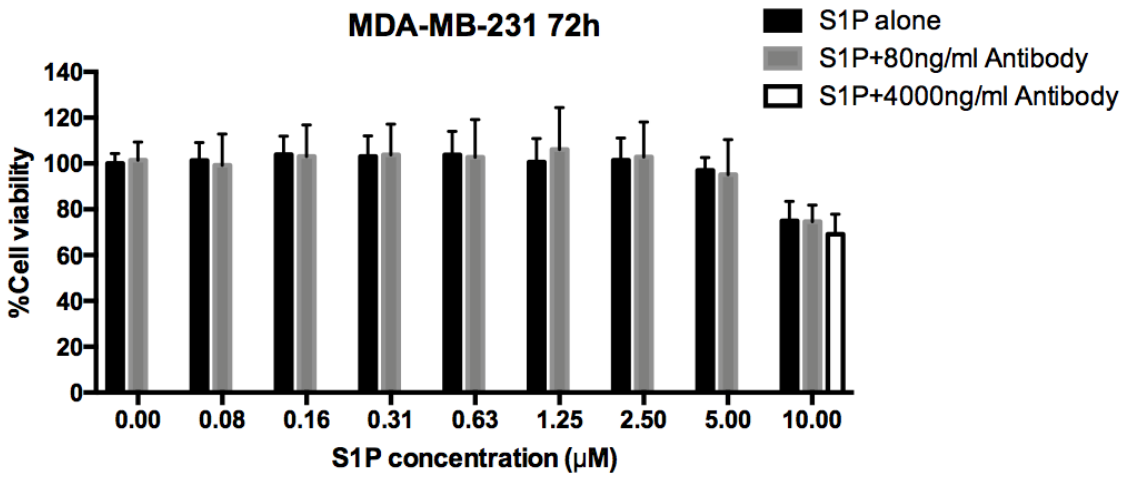
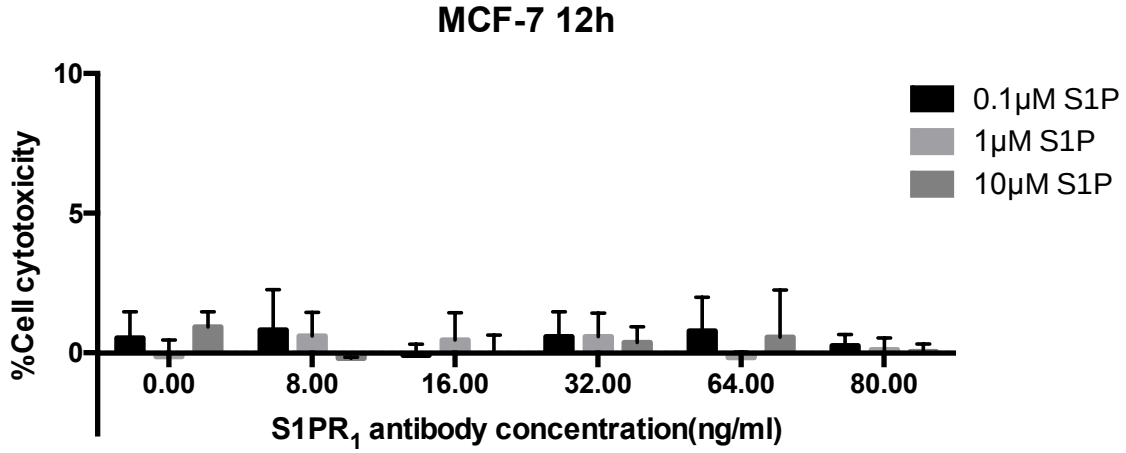
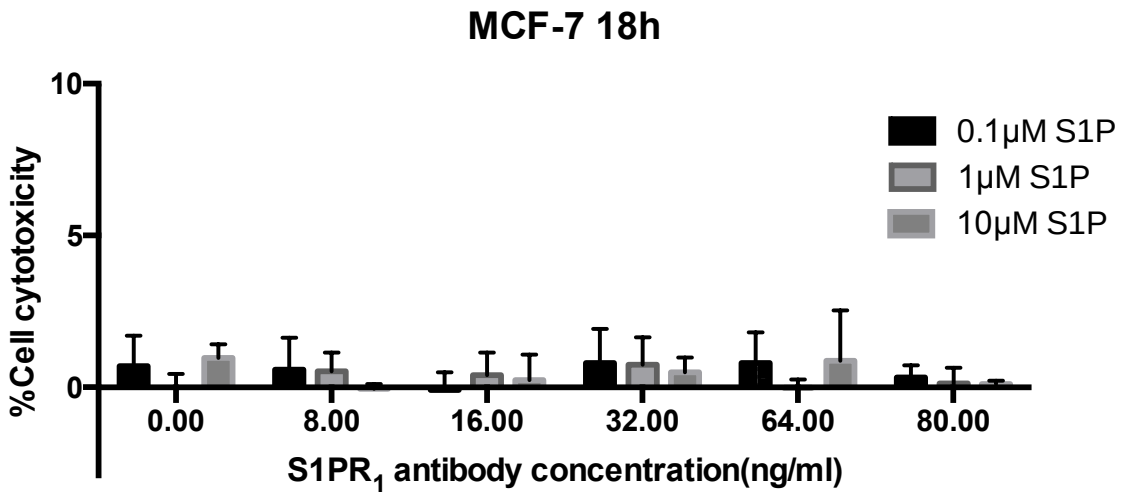
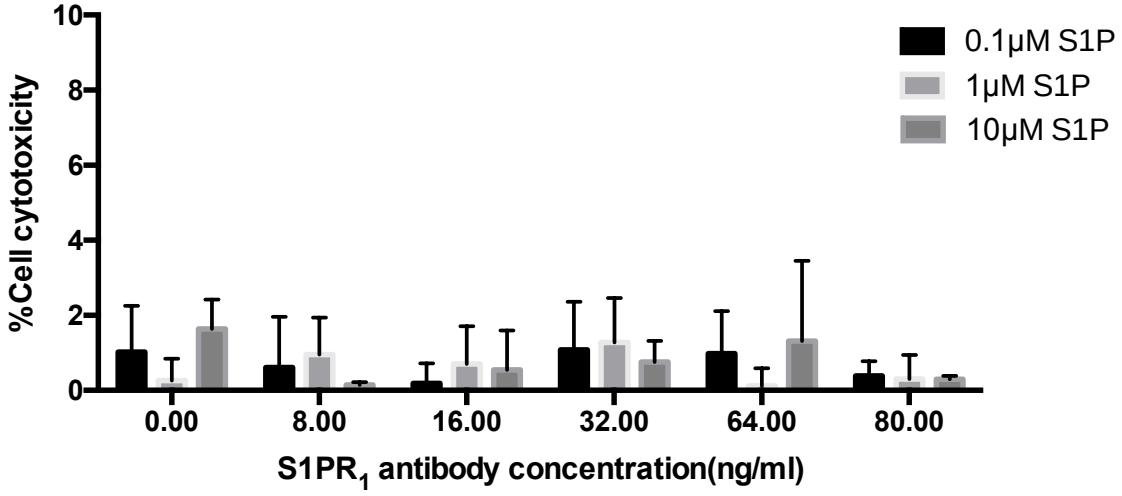
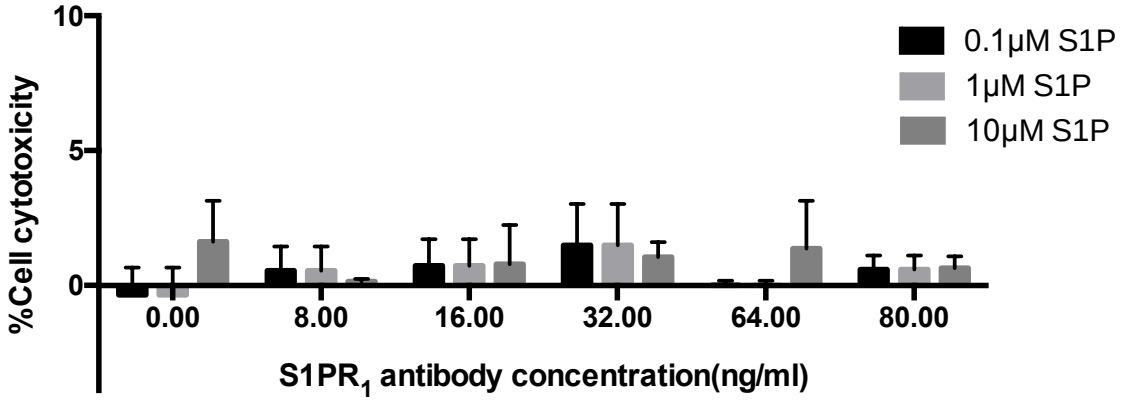


Figure 13. The effect of S1P combined with $S1PR_1$ antibody on MCF-7, SK-BR-3 and MDA-MB-231 cells were examined using MTT assay. Cells were treated with $S1PR_1$ antibody (80 or 4,000 ng/mL) combined with S1P (0.08 – 10 μM) for up to 72h. DMSO < 1% and PBS were used as vehicle control. Results are presented from triplicate experiments on different occasions. Two-way ANOVA with Sidak analysis was used to analyze the significance between cells treated with S1P combined with $S1PR_1$ antibody and cells treated with S1P alone. The data are reported as mean ± SD (N=3), significance was set as $P \leq 0.05$. Note: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$.




MCF-7 18h
%Cell cytotoxicity
10
5
0
0.1μM S1P
1μM S1P
10μM S1P
0.00
8.00
16.00
32.00
64.00
80.00
S1PR1 antibody concentration(ng/ml)


MCF-7 24h
%Cell cytotoxicity
10
8
6
4
2
0
0.1μM S1P
1μM S1P
10μM S1P
0.00
8.00
16.00
32.00
64.00
80.00
S1PR1 antibody concentration(ng/ml)


MCF-7 48h
%Cell cytotoxicity
10
5
0
0.1μM S1P
1μM S1P
10μM S1P
0.00
8.00
16.00
32.00
64.00
80.00
S1PR1 antibody concentration(ng/ml)

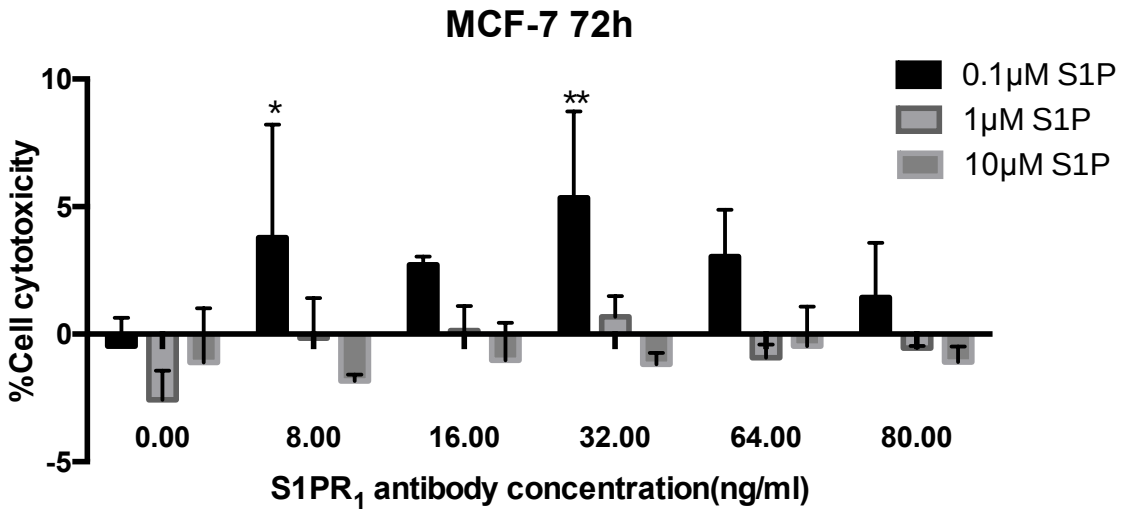
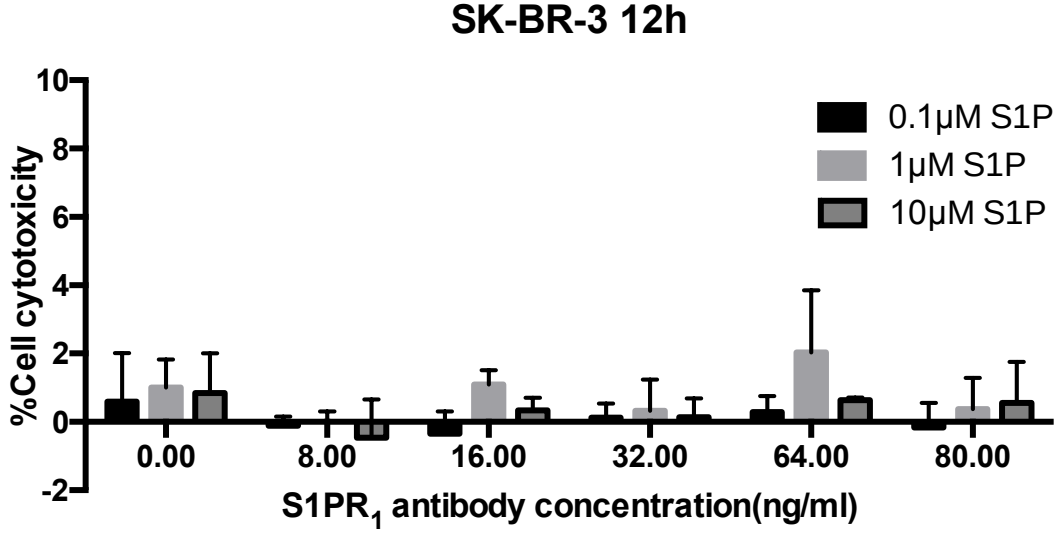
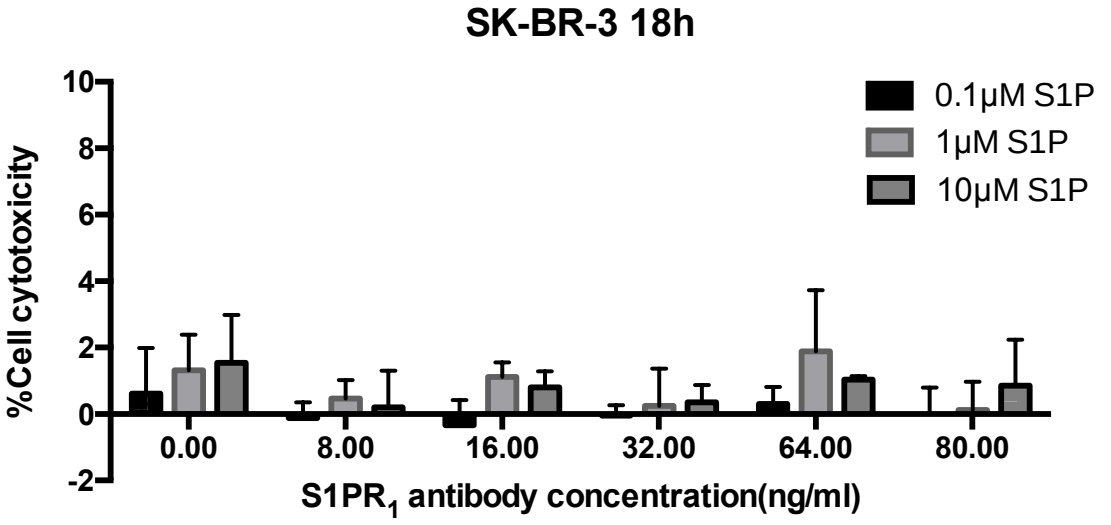
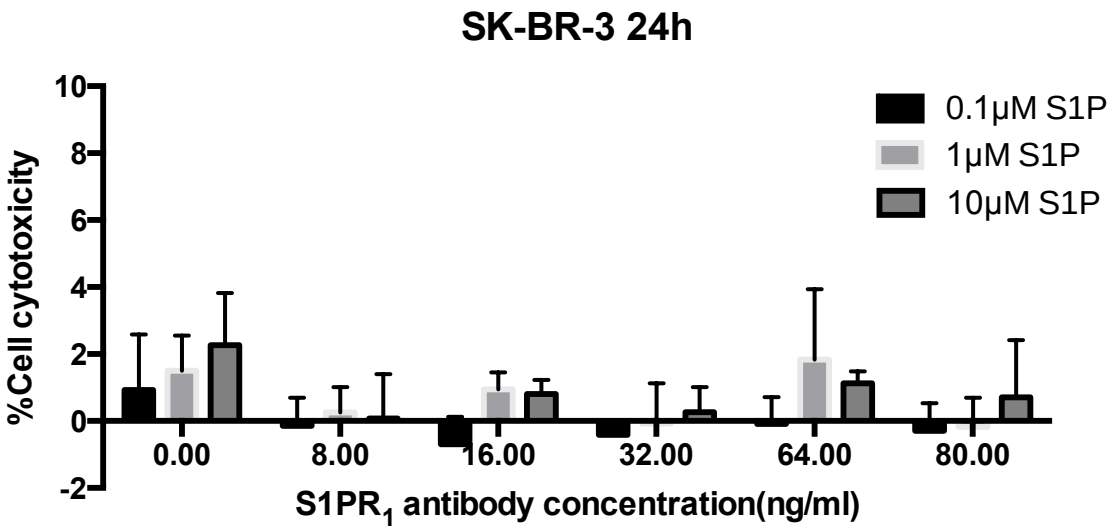


Figure 14. Cytotoxic effect of S1P in combination with $S1PR_1$ antibody on MCF-7 cells with CellTox™ Green Cytotoxicity Assay. Cells were exposed to S1P (0.1 μM, 1 μM, or 10 μM) with $S1PR_1$ antibody (8 – 80 ng/ml) for up to 72h. DMSO < 1% and PBS were used as vehicle control. Results were from duplicate independent experiments. Two-way ANOVA with Sidak analysis was used to analyze the significance between cells treated with S1P combined with $S1PR_1$ antibody and cells treated with S1P alone. The data are reported as mean ± SD (N=2), significance was set as $P \leq 0.05$. Note: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$.




SK-BR-3 18h
0.1μM S1P
1μM S1P
10μM S1P
%Cell cytotoxicity
10
8
6
4
2
0
-2
0.00
8.00
16.00
32.00
64.00
80.00
S1PR1 antibody concentration(ng/ml)


SK-BR-3 24h
0.1μM S1P
1μM S1P
10μM S1P
%Cell cytotoxicity
10
8
6
4
2
0
-2
0.00
8.00
16.00
32.00
64.00
80.00
S1PR1 antibody concentration(ng/ml)

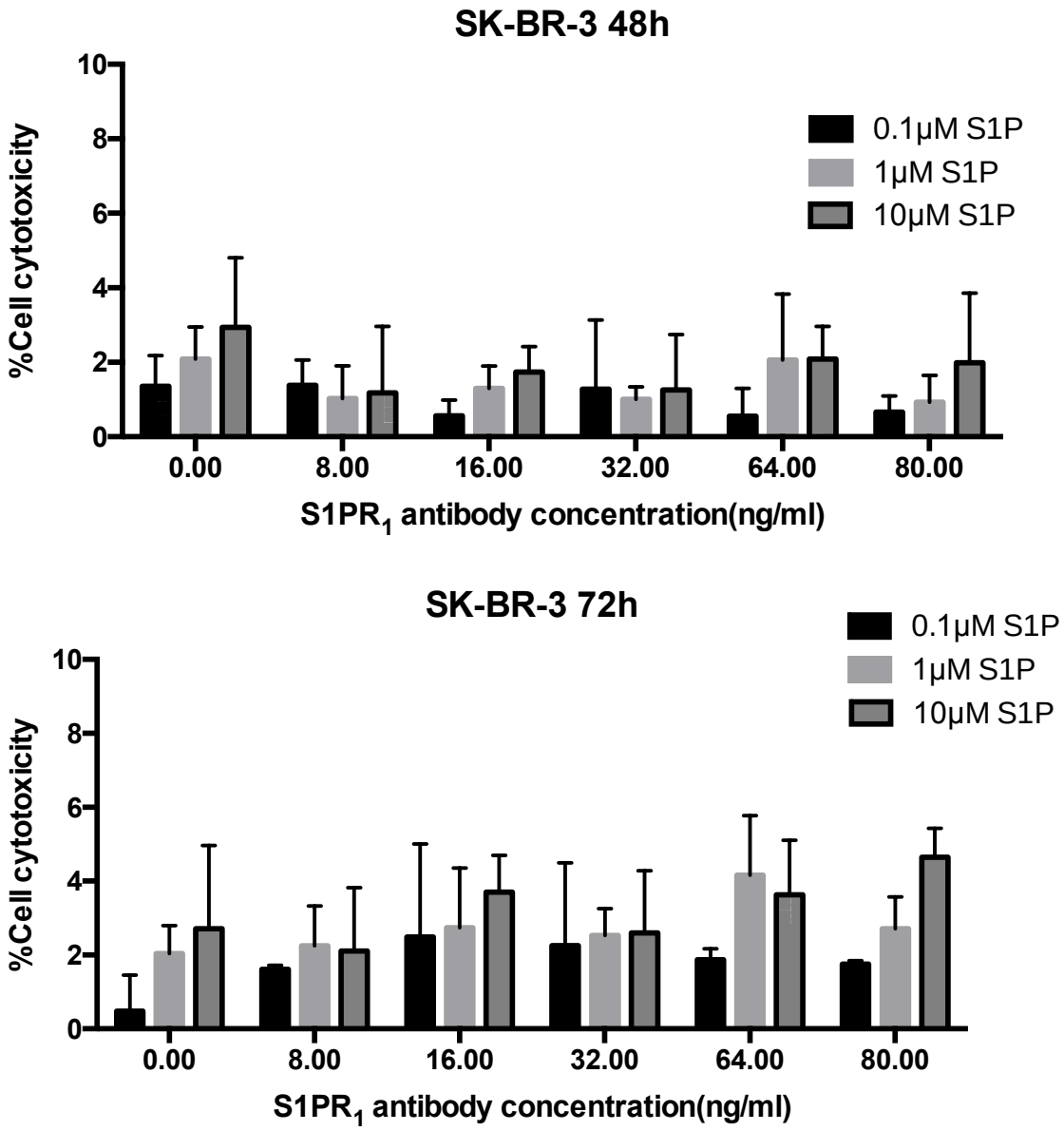
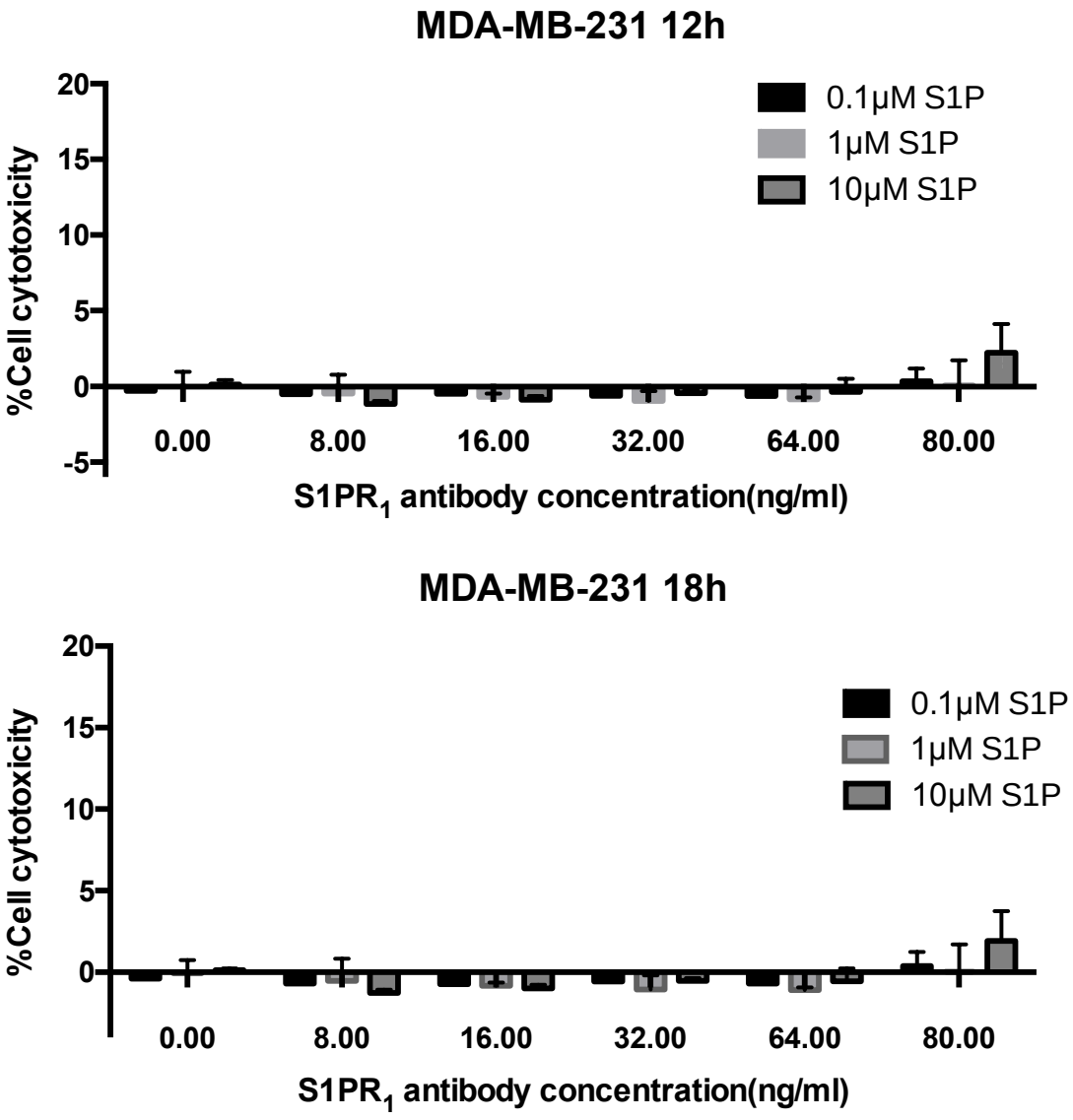


Figure 15. Cytotoxic effect of S1P in combination with $S1PR_1$ antibody on SK-BR-3 cells with CellTox™ Green Cytotoxicity Assay. Cells were exposed to S1P (0.1 μM, 1 μM, or 10 μM) with $S1PR_1$ antibody (8 – 80 ng/ml) for up to 72h. DMSO < 1% and PBS were used as vehicle control. Results were from duplicate independent experiments. Two-way ANOVA with Sidak analysis was used to analyze the significance between cells treated with S1P combined with $S1PR_1$ antibody and cells treated with S1P alone. The data are reported as mean ± SD (N=2), significance was set as $P \leq 0.05$. Note: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$.


MDA-MB-231 12h
0.1μM S1P
1μM S1P
10μM S1P
%Cell cytotoxicity
20
15
10
5
0
-5
0.00
8.00
16.00
32.00
64.00
80.00
S1PR1 antibody concentration(ng/ml)
MDA-MB-231 18h
0.1μM S1P
1μM S1P
10μM S1P
%Cell cytotoxicity
20
15
10
5
0
0.00
8.00
16.00
32.00
64.00
80.00
S1PR1 antibody concentration(ng/ml)

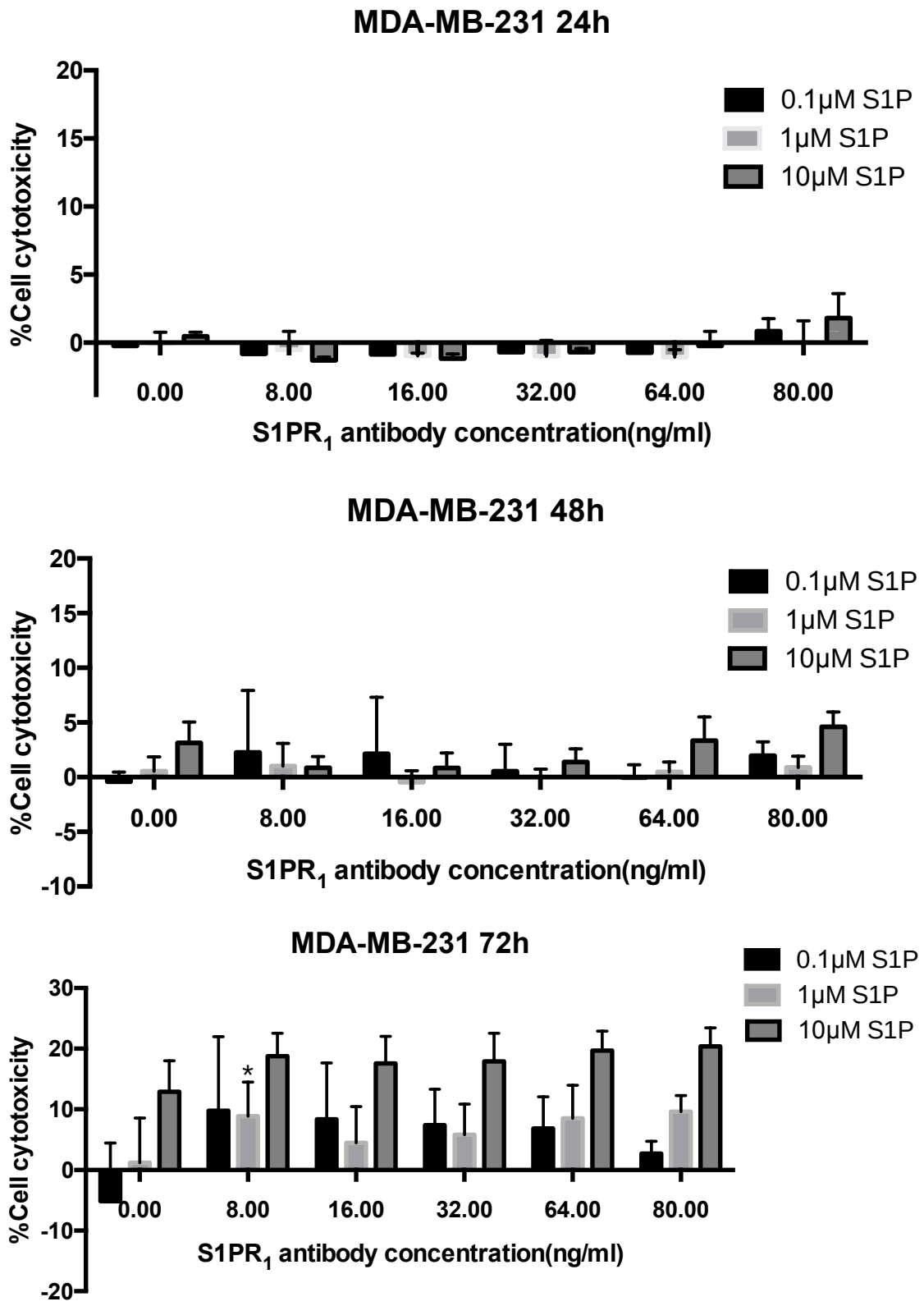
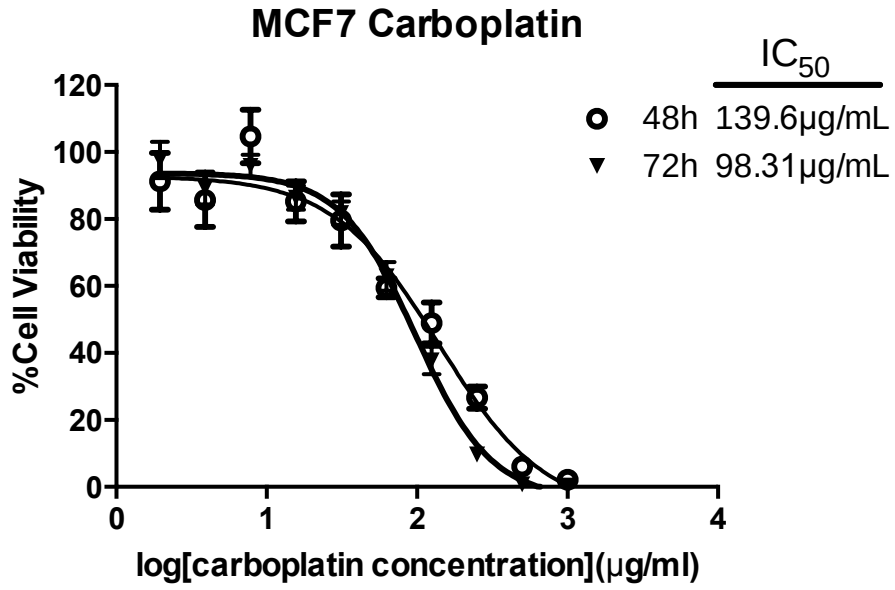


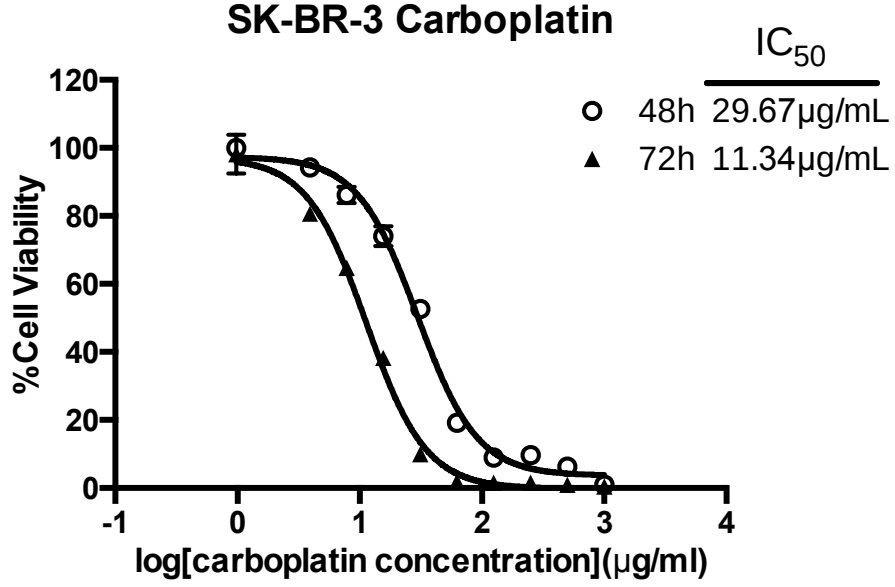
Figure 16. Cytotoxic effect of S1P in combination with $S1PR_1$ antibody on MDA-MB-231 cells with CellTox™ Green Cytotoxicity Assay. Cells were exposed to S1P (0.1 μM, 1 μM, or 10

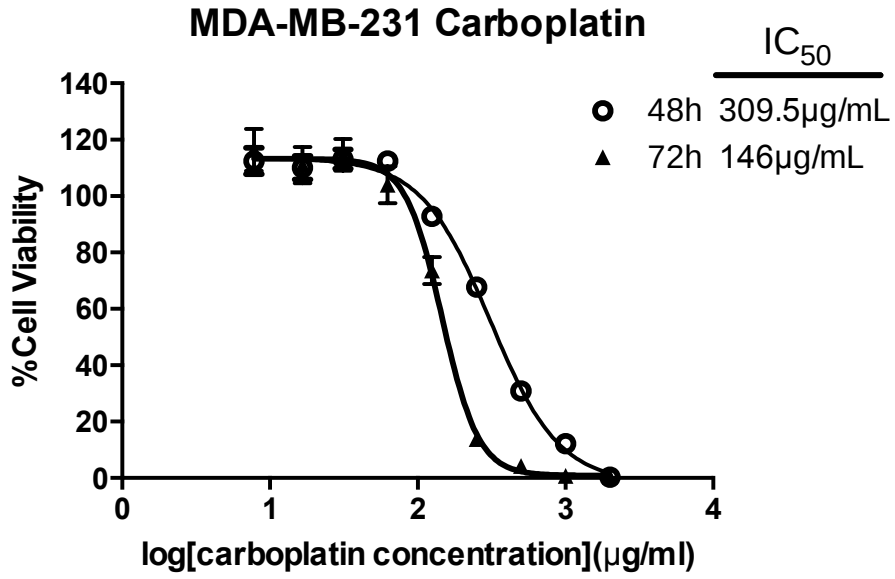
μM) with $S1PR_1$ antibody (8 – 80 ng/ml) for up to 72h. DMSO < 1% and PBS were used as vehicle control. Results were from duplicate independent experiments. Two-way ANOVA with Sidak analysis was used to analyze the significance between cells treated with S1P combined with $S1PR_1$ antibody and cells treated with S1P alone. The data are reported as mean ± SD (N=2), significance was set as $P \leq 0.05$. Note: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$.

5.3 IC_{50} value of carboplatin

The cytotoxic effect of carboplatin was determined in breast cancer cell lines SK-BR-3 and MDA-MB-231 using the MTT assay. As expected, there was a dose-dependent cell killing effect. The IC_{50} of carboplatin was determined to be 376 μM (48h) and 265 μM (72 h) against MCF-7 cells, 80.0 μM (48 h) and 30.6 μM (72 h) against SK-BR-3 cells, and 834 μM (48 h) and 294 μM (72 h) against MDA-MB-231 cells, respectively. Previous studies have shown that the IC_{50} of carboplatin was 62.19 μM (72h) against MCF-7 cells[149], 45.43 μM (72h) against SK-BR-3 cells[149], and 269 μM (72h) against MDA-MB-231 cells[150]. The IC_{50} values of carboplatin of SK-BR-3 and MDA-MB231 cells in my experiment were quite close with the published results.[149,150] However, the values of the MCF-7 cells are quite different – 265 μM from my experiment and 62.19 μM (72h) from the published results. The divergence may be due to the genetic drift and phenotype drift between the MCF-7 cells in my experiment and the one used in publication, the differences in preparing carboplatin solutions, the methods applied to calculate the IC_{50} value or the range of concentrations used to assess the IC_{50} of carboplatin between my experiment and the publication. Nevertheless, the IC_{50} value of carboplatin in these three cell lines is extremely high compared to the reported IC_{50} of carboplatin towards several cell lines derived from ovarian cancer patients, which is around 1 μM for pre-chemotherapy cell lines and 5 μM for post-chemotherapy cell lines.[151] This implies that carboplatin might not be the first choice for treating luminal A, HER2 or triple-negative subtype breast cancer, although it is approved for advanced-stage breast cancer. Based on the IC_{50} values, 6 to 7 concentrations were decided in further combination experiments with S1P or $S1PR_1$ antibody.


MCF7 Carboplatin
IC50
48h 139.6µg/mL
72h 98.31µg/mL
%Cell Viability
120
100
80
60
40
20
0
0
1
2
3
4
log[carboplatin concentration](µg/ml)


SK-BR-3 Carboplatin
IC50
48h 29.67µg/mL
72h 11.34µg/mL
%Cell Viability
120
100
80
60
40
20
0
-1
0
1
2
3
4
log[carboplatin concentration](µg/ml)



	IC$_{50}$ (95% CI) (μM)		
Cell types	**MCF-7**	**SK-BR-3**	**MDA-MB-231**
48h	376 (219.8-644.5)	80 (72.8-87.8)	834 (738.5-942.6)
72h	265 (221.9-316.4)	30.6 (28.0-33.3)	394 (355.3-436.4)

Figure 17. Dose responses curves show IC$_{50}$ of carboplatin on reduction of breast cancer cell lines MCF-7, SK-BR-3, and MDA-MB-231 cell viability. Error bars represent mean values ± standard deviation of three replicates on three different occasions. PBS was used as vehicle control.

5.4 S1PR$_1$ antibody sensitized the breast cancer cells to carboplatin at high concentrations

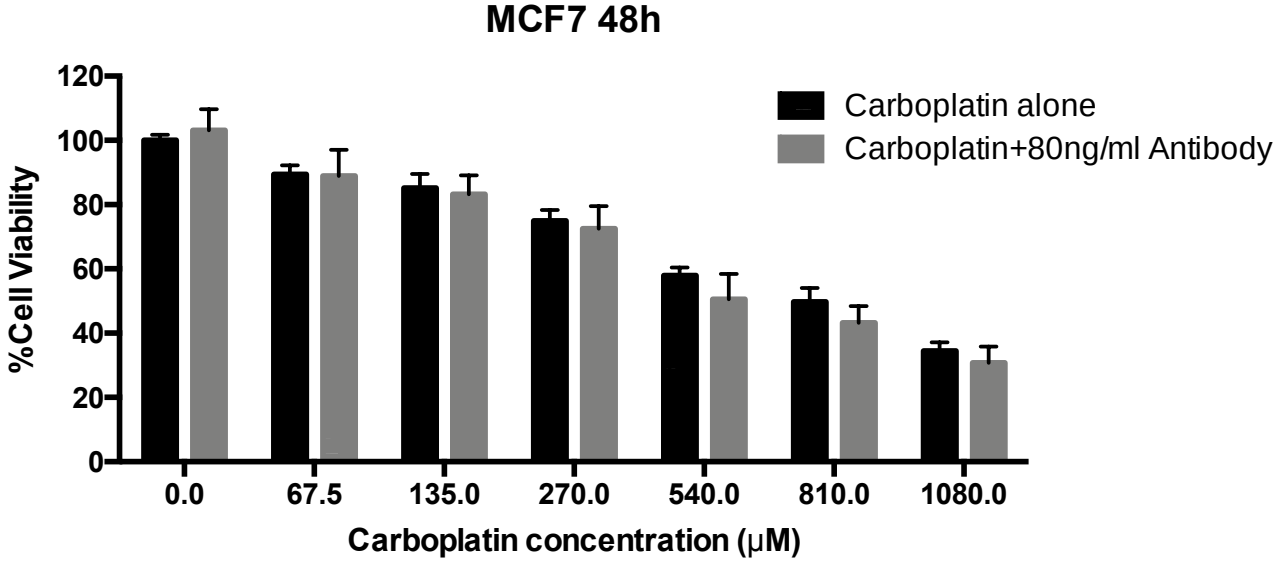
In order to assess the effects of co-administrated S1PR$_1$ antibody with carboplatin in MCF-7, SK-BR-3 and MDA-MB-231 cells, I evaluated the effect on cell viability of carboplatin alone and combined with S1PR$_1$ antibody using the MTT assay. Two S1PR$_1$ antibody concentrations were used in this experiment. First, 80 ng/mL S1PR$_1$ antibody was chosen to determine whether there was synergistic effect between carboplatin and S1PR$_1$ antibody. Compared to cells treated with carboplatin and cells treated with carboplatin combined with 80 ng/ml S1PR$_1$ antibody, no significant difference was observed for the effect on cell viability in

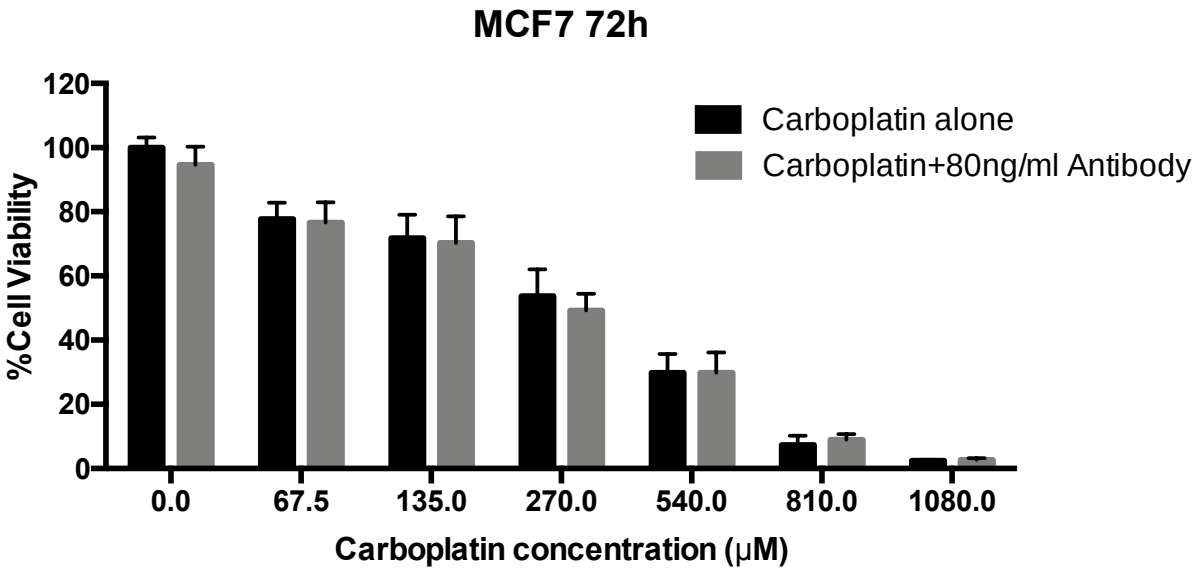
MCF-7, SK-BR-3 and MDA-MB-231 cells (Figure 18). Therefore, low concentrations of $S1PR_1$ antibody (such as 80 ng/mL) cannot enhance the cytotoxic effect of carboplatin in these three cell lines. Therewith $S1PR_1$ antibody (4,000 ng/mL), which alone decreased cell viability in both SK-BR-3 and MDA-MB-231 cells, was tested in the SK-BR-3 and MDA-MB-231 cells in combination with carboplatin. Because of the shortage in funding, no experiments were conducted in the MCF-7 cells. For the SK-BR-3 and MDA-MB-231 cells, I chose a concentration of carboplatin significantly lower than the IC_{50} (4 μM for SK-Br-3 and 68 μM for MDA-MB-231), which does not impose any cytotoxicity to the cells. The reason for this type of selection is to ensure that this research would lead to further *in vivo* and even clinically meaningful studies if the $S1PR_1$ antibody indeed sensitized breast cancer cells to carboplatin as a recent study shows using FTY720 to block $S1PR_1$ receptor on neuroblasma cells sensitizes cells to etoposide, a chemo-drug.[152]. In addition, I chose a high concentration of carboplatin, significantly higher than the IC_{50} for these two cell lines (540 μM for SK-Br-3 and 1080 μM for MDA-MB-231). As shown in Figure 19, only marginal difference was observed after 48 h and 72 h treatment with 4.2 μM carboplatin and 4.2 μM carboplatin combined with $S1PR_1$ antibody in the SK-BR-3 cells. For the MDA-MB-231 cells, the co-administration of $S1PR_1$ antibody and low concentration of carboplatin decreased the cell viability by more than 35% compared to carboplatin alone ($p \leq 0.0001$). This suggested that 4,000 ng/mL $S1PR_1$ antibody enhanced the cytotoxic effect of carboplatin.

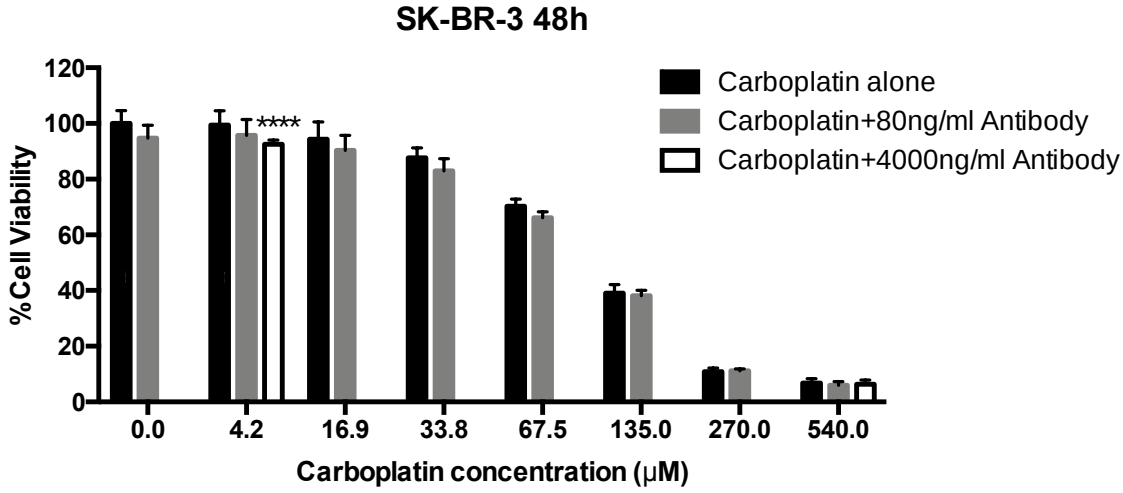
As shown in Figure 19, the enhancement effect of 4,000 ng/mL $S1PR_1$ antibody on carboplatin is more notable in triple-negative subtype MDA-MB-231 cells than in HER2 subtype SK-BR-3 cells. This might due to the gene expression difference between these two cell lines. It is possible that 4,000 ng/mL $S1PR_1$ antibody can enhance the cytotoxic effect of carboplatin in triple-negative breast cancer cells more obviously than other breast cancer subtypes. In order to demonstrate this conjecture, more triple-negative subtype cell lines should be used in the future studies.

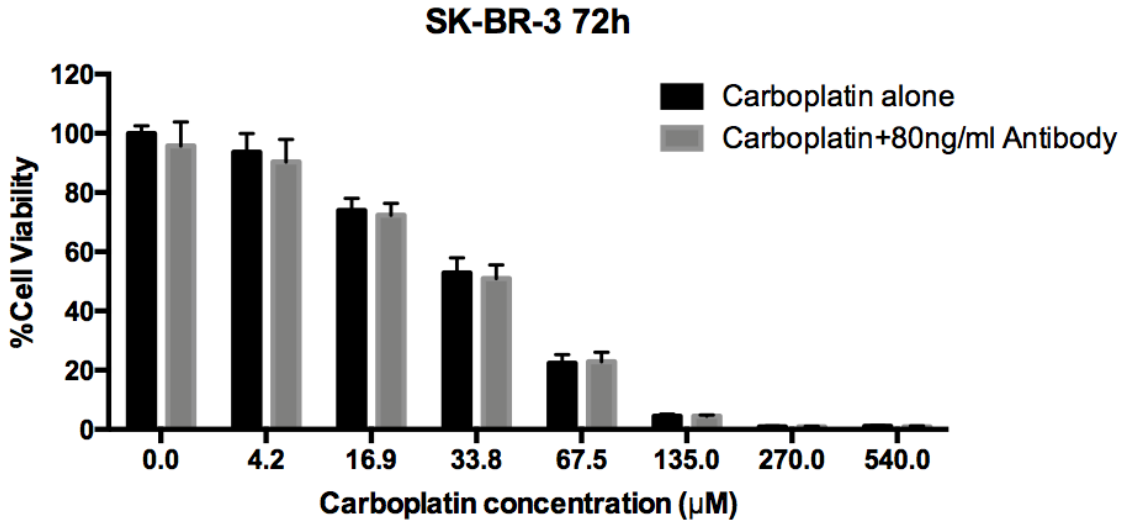
The enhanced cytotoxic effect of carboplatin by the $S1PR_1$ antibody might be due to $S1PR_1$ antibody interference with the SK1/S1P/$S1PR_1$ signaling axis and suppression of the NF-κB/IL-6/STAT3 loop in breast cancer cell lines, as observed for FTY720's ability to sensitize

breast cancer cells to doxorubicin.[18] In addition, co-administration of $S1PR_1$ antibody with carboplatin may help to delay tumor recurrent and carboplatin drug resistance in breast cancer. In breast cancer, carboplatin was found to induce the expression of glutathione S-transferase omega 1 (GSTO1), which can catalyze the production of glutathione that contributes to drug resistance.[153] Then GSTO1 can lead to calcium release to the cytoplasm.[153] Through activation of STAT3 pathway, it can increase the expression of pluripotency factors and breast cancer stem cell enrichment, increasing chemo-resistance.[153] At the same time, blocking $S1PR_1$ with its antibody may inhibit the calcium release from endoplasmic reticulum, therefore, decreasing the chemo-resistance of carboplatin in breast cancer and improving the clinical outcome.




MCF7 72h
Carboplatin alone
Carboplatin+80ng/ml Antibody
%Cell Viability
120
100
80
60
40
20
0
0.0
67.5
135.0
270.0
540.0
810.0
1080.0
Carboplatin concentration (μM)


SK-BR-3 48h
Carboplatin alone
Carboplatin+80ng/ml Antibody
Carboplatin+4000ng/ml Antibody
****
%Cell Viability
120
100
80
60
40
20
0
0.0
4.2
16.9
33.8
67.5
135.0
270.0
540.0
Carboplatin concentration (μM)


SK-BR-3 72h
Carboplatin alone
Carboplatin+80ng/ml Antibody
%Cell Viability
120
100
80
60
40
20
0
0.0
4.2
16.9
33.8
67.5
135.0
270.0
540.0
Carboplatin concentration (μM)

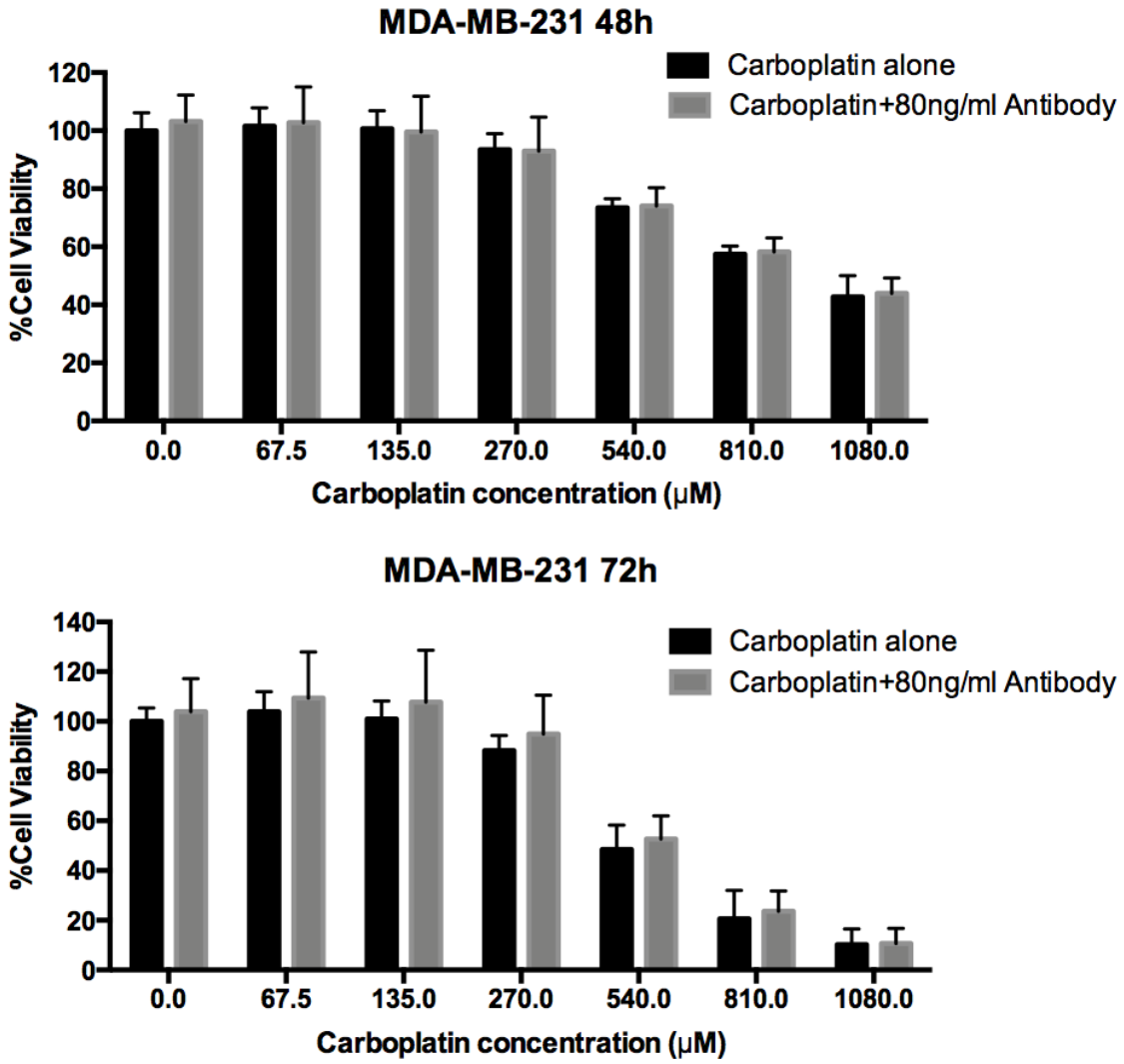


Figure 18. Toxic effect of carboplatin in combination with 80 ng/mL $S1PR_1$ antibody on MCF-7, SK-BR-3, and MDA-MB-231 cells for 48 h or 72 h tested with MTT assay. Cells treated with solvent as a negative control. Results were from triplicate independent experiments. Two-way ANOVA with Sidak analysis was used to analyze the significance between cells treated with carboplatin combined with $S1PR_1$ antibody and cells treated with carboplatin alone. The data are reported as mean ± SD (N=3), significance was set as $P \leq 0.05$. Note: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$.

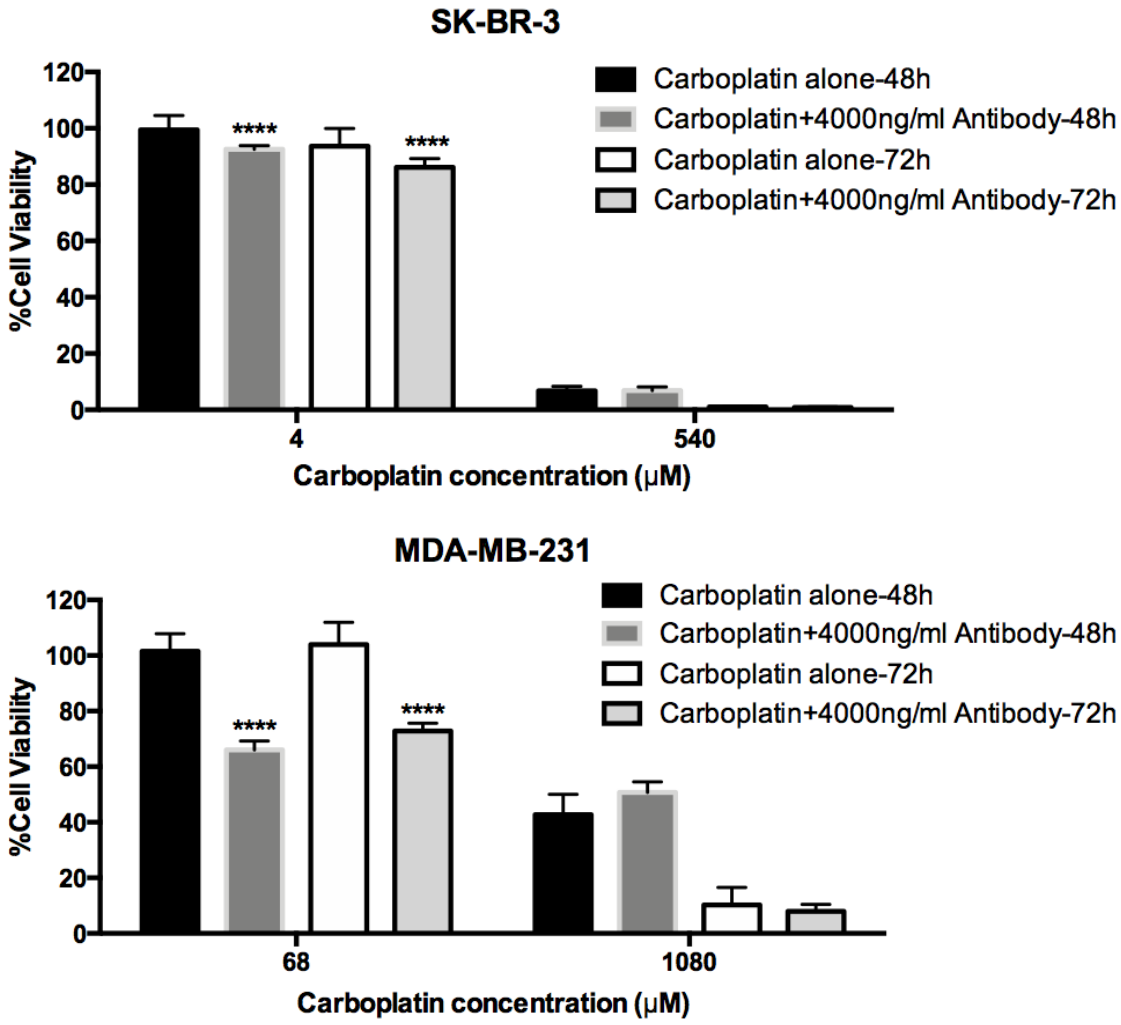
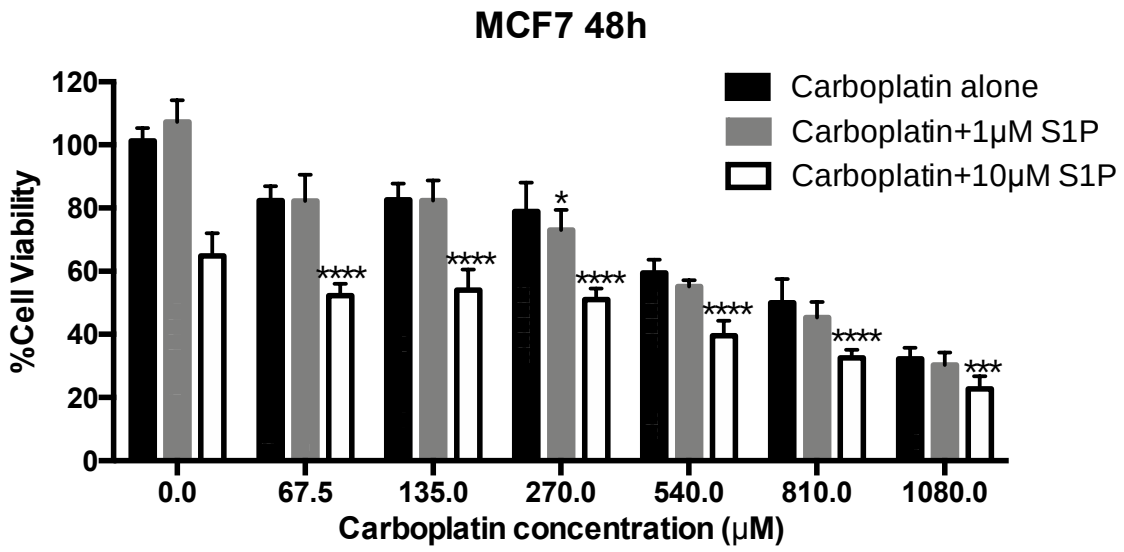
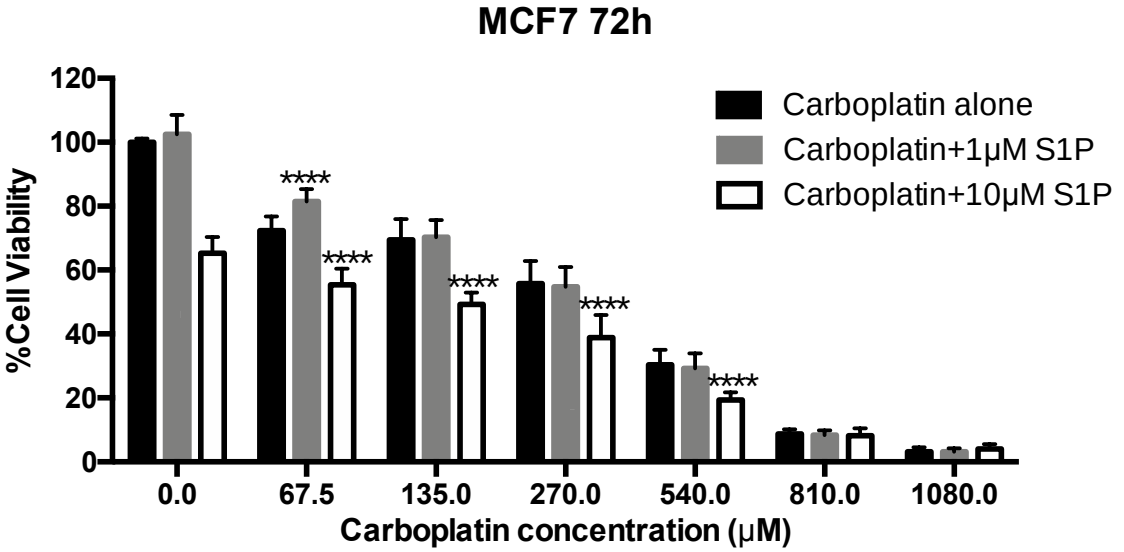


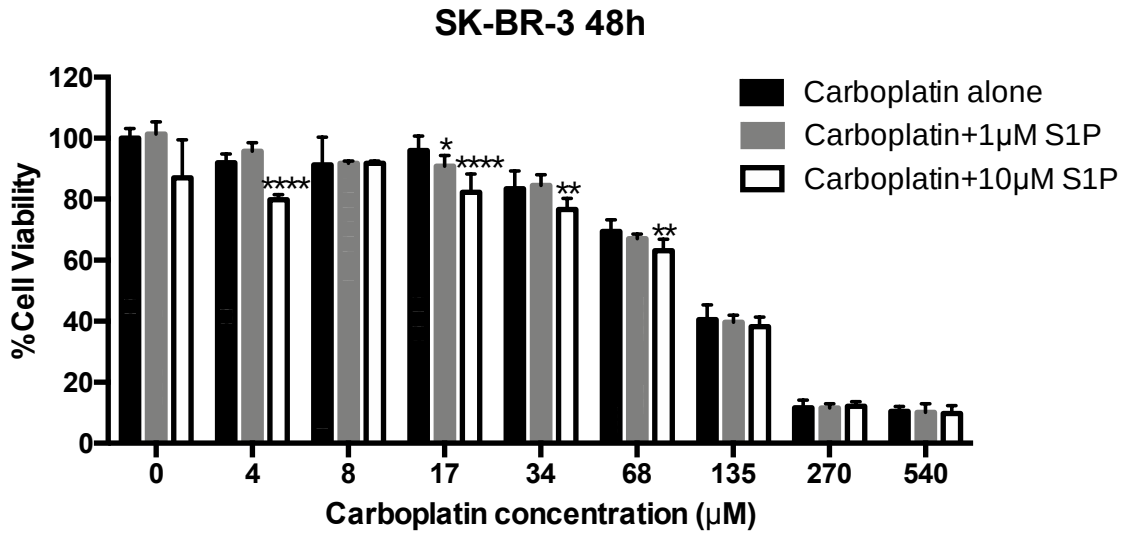
Figure 19. Toxic effect of carboplatin in combination with 4,000 ng/mL $S1PR_1$ antibody on SK-BR-3 and MDA-MB-231 cells for 48 h or 72 h tested with MTT assay. Cells treated with solvent as a negative control. Results were from triplicate independent experiments. Two-way ANOVA with Sidak analysis was used to analyze the significance between cells treated with carboplatin combined with $S1PR_1$ antibody and cells treated with carboplatin alone. The data are reported as mean ± SD (N=3), significance was set as $P \leq 0.05$. Note: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$.

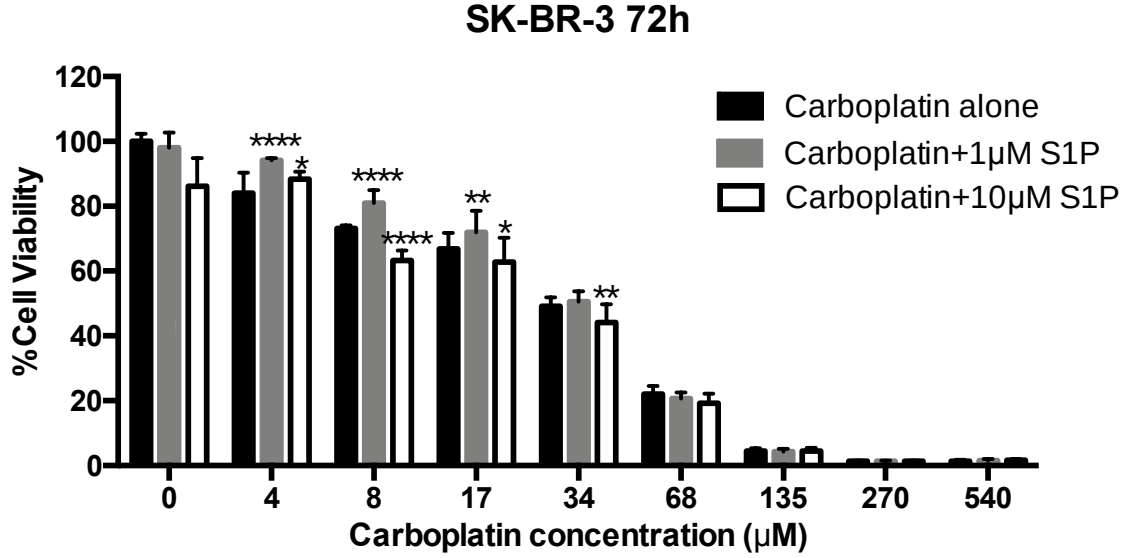
5.5 S1P could not enhance the cytotoxic effect of carboplatin in breast cancer cells

The effects of carboplatin alone and combined with S1P on cell viability in MCF-7, SK-BR-3 and MDA-MB-231 cells were assessed using the MTT assay. The concentrations of S1P were 1 μM and 10 μM in this experiment. Normally, the concentration of S1P in plasma is around 0.2 – 0.9 μM. Therefore, 1 μM concentration, which is close to the normal plasma level of S1P, was chosen. Concentration of 10 μM was used because that S1P selectively induced cell apoptosis in breast cancer cells MCF-7 and exhibited synergistic effects with chemotherapy drugs towards breast cancer MCF-7 and MDA-MB-361 cells at a concentration higher than 1 μM, especially at 10 μM [70,141]. As shown in Figure 20, neither 1 μM nor 10 μM of S1P was able to sensitize MCF-7, SK-BR-3 and MDA-MB-231 cells to carboplatin treatment. However, 10 μM S1P decreased the cell viability of the MCF-7, SK-BR-3 and MDA-MB-231 cells by approximately 30%, 15% and 30%, respectively, at both 48 h and 72 h treatments. For the three cell lines, when carboplatin concentration was less than the IC_{50} values, the effect of the co-administration of 10 μM S1P and carboplatin on cell viability was dominated by S1P. Whereas the carboplatin concentration was increased above the IC_{50} values, the effect of the co-administration of S1P (concentration: 10 μM) and carboplatin on cell viability was controlled by carboplatin. Therefore, contrary to our previous studies that S1P can increase the cytotoxic activities of docetaxel, doxorubicin and cyclophosphamide, co-administration of high concentrations of S1P does not improve the cytotoxic activity of carboplatin. The reason for this is still unknown. Docetaxel, doxorubicin, cyclophosphamide and carboplatin have different mechanisms of killing cancer cells. Docetaxel is an anti-mitotic agent through stabilized microtubule[154]; doxorubicin is an anthracycline antibiotic that can intercalate into DNA and interrupt the function of topoisomerase-II-mediated DNA repair[155]; cyclophosphamide is a nitrogen mustard alkylating agent that prevents DNA synthesis and RNA transcription through forming complex with DNA; and carboplatin is a DNA alkylating agent that can covalently link to DNA bases and form DNA adducts. In order to find the mechanisms on how S1P enhances the cytotoxic effect of some agents used in chemotherapy, more chemotherapy drugs need to be assessed.


MCF7 48h
Carboplatin alone
Carboplatin+1μM S1P
Carboplatin+10μM S1P
%Cell Viability
120
100
80
60
40
20
0
*
****
****
****
****
****
***
0.0
67.5
135.0
270.0
540.0
810.0
1080.0
Carboplatin concentration (μM)


MCF7 72h
Carboplatin alone
Carboplatin+1μM S1P
Carboplatin+10μM S1P
%Cell Viability
120
100
80
60
40
20
0
****
****
****
****
****
0.0
67.5
135.0
270.0
540.0
810.0
1080.0
Carboplatin concentration (μM)


SK-BR-3 48h
Carboplatin alone
Carboplatin+1μM S1P
Carboplatin+10μM S1P
%Cell Viability
120
100
80
60
40
20
0
****
*
****
**
**
0
4
8
17
34
68
135
270
540
Carboplatin concentration (μM)


SK-BR-3 72h
Carboplatin alone
Carboplatin+1μM S1P
Carboplatin+10μM S1P
%Cell Viability
120
100
80
60
40
20
0
****
*
****
****
**
*
**
0
4
8
17
34
68
135
270
540
Carboplatin concentration (μM)

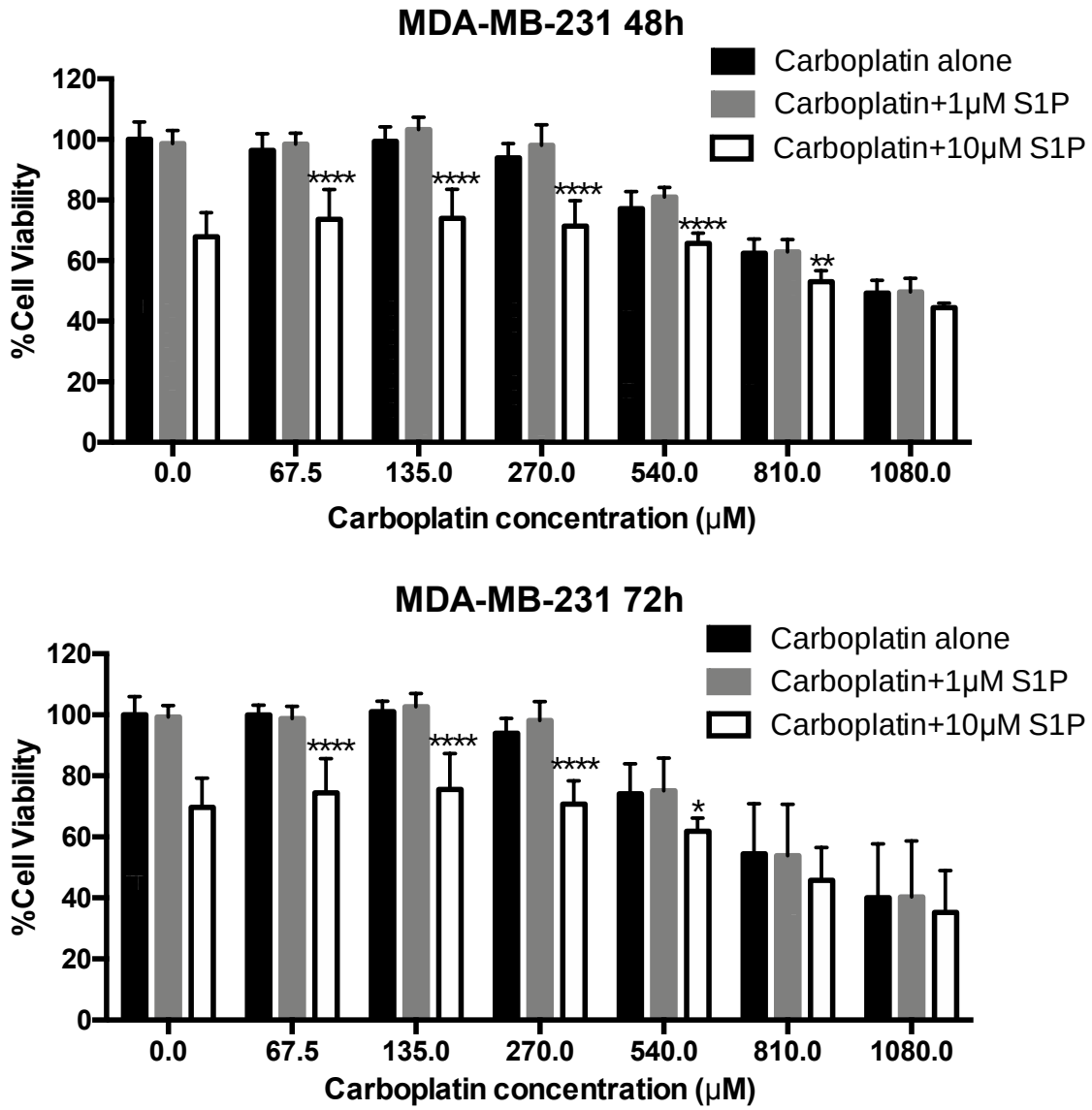


Figure 20. Percent cell viability of MCF-7, SK-BR-3 and MDA-MB-231 cells after exposure to carboplatin alone or combined with S1P. Cells were treated with S1P (0.08 – 10 μM) combined with carboplatin (4.2 – 1080 μM). DMSO < 1% and PBS were used as vehicle control. Results were obtained in three independent experiments. Two-way ANOVA with Sidak analysis was used to analyze the significance between cells treated with carboplatin combined with S1P and cells treated with carboplatin alone. The data are reported as mean ± SD (N=3), significance was set as $P \leq 0.05$. Note: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$.

6 Limitations and conclusions

In this study, we showed that the $S1PR_1$ antibody exhibited cytostatic effects towards luminal A subtype MCF-7 cell line, HER2 subtype SK-BR-3 cell line, and triple-negative subtype MDA-MB-231 cell line. Co-administration of 4,000 ng/mL of the $S1PR_1$ antibody not only potentiated the cytotoxicity of carboplatin towards cell line MDA-MB-231 but also increased the anti-proliferative effect of S1P towards cell line SK-BR-3. Furthermore, co-administration of S1P did not improve the response of the MCF-7, SK-BR-3 and MDA-MB-231 cells towards carboplatin treatment.

There are, however, some limitations in this study. First, the cytotoxic effect of co-administration of 4,000 ng/mL $S1PR_1$ antibody with S1P and carboplatin, respectively, was not assessed in MCF-7 cells because of funding limitations. Second, only two different concentrations of carboplatin were used to assess the co-administration of 4,000 ng/mL $S1PR_1$ antibody on SK-BR-3 and MDA-MB-231 cells. Third, when assessing the effect of 4,000 ng/mL $S1PR_1$ antibody and carboplatin, no 4,000 ng/mL $S1PR_1$ antibody alone was tested at the same time to act as a negative control. Therefore, in the future, cytotoxicity assay should be conducted on different concentrations of carboplatin with 4,000 ng/mL $S1PR_1$ antibody in these three cell lines, using carboplatin and 4,000 ng/mL $S1PR_1$ antibody alone as controls.

7 Future work

The mechanisms of the cytostatic effect of $S1PR_1$ antibody should be focused in the future. To discover the mechanism, the following questions should be studied. First, the expression levels of S1PRs on the cell surface should be compared with normal breast cells. This can be done by extracting different S1PRs mRNA from different breast cancer cells lines and normal breast cancer cells, then performing quantitative RT-PCR. Second, the subcellular localization of S1PRs inside the cells should be assessed. This can be done with the help of laser scanning confocal microscopy (LSCM). Third, it should be determined whether the cytostatic effect of $S1PR_1$ antibody is related to $S1PR_2$ and $S1PR_3$ on the cell surface. Blocking $S1PR_1$ with its antibody on the cell surface, S1P produced by the cells themselves can bind to

$S1PR_2$ to reduce cell viability or through the influx into cells and act as a second messenger to elude downstream reactions. Thus, studies are needed to verify whether $S1PR_1$ antibody would have effect on S1P-$S1PR_2$ and S1P-$S1PR_3$ signaling in human breast cancer cells. $S1PR_2$ antibody or antagonists such as JTE-013[75] could be used to assess whether the cytostatic effect of S1P involves downstream reactions of $S1PR_2$ on the cell surface. Fourthly, the synthetic rate and degradation rate of S1P should be assessed in breast cancer cells before and after treating $S1PR_1$ antibody as it may affect the synthesis and degradation rate of S1P in breast cancer cells *in vitro*.

In addition, aggressive breast cancer cell lines should be applied in the cytotoxic study of 4,000 ng/mL $S1PR_1$ antibody combined with carboplatin. Because in the study, it shows that 4,000 ng/mL $S1PR_1$ antibody enhanced the cytotoxic effect of carboplatin in triple-negative subtype MDA-MB-231 cells and no enhancement was observed in HER-2 positive subtype SK-BR-3 cells. Therefore, 4,000 ng/mL $S1PR_1$ antibody may enhance the cytotoxic effect of carboplatin in the more aggressive breast cancer cell types such as triple-negative breast cancer subtype. In addition, co-administration of 4,000 ng/mL $S1PR_1$ antibody with other chemo-drugs such as docetaxel, doxorubicin and cyclophosphamide can be tested in various breast cancer subtypes.

Lastly, the gene expression profiles of normal breast cells and different breast cancer cell lines should be studied with the help of the Cancer Genome Project and the Cancer Cell Line Encyclopedia to link the relationship with gene expression and cell reaction against $S1PR_1$ antibody, carboplatin and these two combinations. Studying the gene expression profiles of normal breast cells and breast cancer cells can also help to explain why S1P can enhance the cytotoxic effect of docetaxel, doxorubicin and cyclophosphamide in MDA-MB-231 cells and MCF-7 cells, but it cannot enhance the cytotoxic effect of carboplatin.

8 References

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